THE ROLE OF PROSTAGLANDIN SYNTHASE II (PGS-2) IN THE IMMUNOPATHOGENESIS OF NOD DIABETES

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1996

ACKNOWLEDGMENTS

I would especially like to thank the chairman of my committee, Dr. Michael Clare-Salzler, for his intellectual guidance and his unflagging support and encouragement. I am also grateful to my committee members, Drs. Ammon Peck, Edward Wakeland, Mark Atkinson and Michael Humphreys-Beher, for their guidance and support in various phases of my research.

I also thank, Drs. Srinivasa Reddy and Harvey Herschman for their helpful advice and technical support. I also appreciate the help from Drs. Linder Wicker, Edward Wakeland, Mark Atkinson and Mary Yui for providing congenic mice, NODscid/scid mice, and many valuable discussions.

I have enjoyed my association with other members of the Dr. Clare-Salzler's lab and members of Dr. Peck's lab, especially Andrea Hofig, Sally Litherland, Janet Cornelius, Jeff Anderson, Chris Robinson, Joanne Johnson-Tardieu and Bertha Moreno-Altamirano and thank them for help and encouragement. Thanks go to Christy Myrick for confirming the PGS-2 promoter sequence and special thanks go to both Sally Litherland and Christy Myrick for helping with thesis editing.

My special thanks go to both Michael and Rita Clare-Salzler for friendship, support and great meals.

I want to thank my parents for their encouragement for my endeavors through the years. Lastly, I am grateful to my wife, for her love and support throughout the years.

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December, 1996

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The non-obese diabetic (NOD) mouse is a well-established animal model of the human autoimmune disease, insulin dependent diabetes (IDD). In NOD mice, both T cells and macrophages (MPs) play an important role in the immunopathogenesis of this disease. The cellular and molecular basis of MP involvement in NOD diabetes, however, is largely unknown. In the present study, we have investigated the expression of prostaglandin synthase II (PGS-2) and PGE-2 production in the NOD MPs and its role in the immunopathogenesis of NOD diabetes.

Our results indicate that 1) MPs from NOD and NOD scid/scid estrus female mice, unlike those of non-autoimmune mice, spontaneously express abundant PGS-2 mRNA and protein. 2) There is a sexual dimorphism in the PGS-2 mRNA and protein expression. Whereas MPs from male NOD mice do not spontaneously express PGS-2, spontaneous PGS-2 mRNA expression is observed at all times in MPs of NOD female mice, while PGS-2 protein expression is observed only during estrus phase. Our *in vitro* experiments demonstrate that female steroid hormones strongly influence PGS-2

expression, whereas male steroid hormones have no effect. 3) PGS-2 expression in NOD MPs is insensitive to IL-10. In our *in vitro* studies, mrIL-10 completely suppressed LPS induced PGS-2 expression in MPs of control mice at 10ng/ml. In contrast, mrIL-10 did not suppress spontaneously expressed PGS-2 in NOD MPs at concentration as high as 500 ng/ml. 4) Different PGS-2 expression in both NOD and normal chromosome 1 congenic mice suggest that PGS-2 could be a candidate gene of diabetes susceptibility for *Idd5*. 5) There is enhanced prostaglandin production by MPs through the constitutive PGS-2 expression. Treating female NOD mice with drugs that block PGS-2 enzymatic activity significantly delays the onset and reduces the incidence of diabetes. 6) There is a general impaired activation and deletion of T cells in NOD mice which is predominantly affected by the NOD MHC molecule H-2^{g7}, and that PGS-2 appears to contribute to a defect in T cell deletion. These data strongly suggest that spontaneous expression of PGS-2 in NOD MPs plays an important role in the immunopathogenesis of diabetes in NOD mice and is a candidate gene for *Idd5*.

CHAPTER 1
INTRODUCTION
Insulin Dependent (type I) Diabetes (IDD)

Insulin-dependent diabetes mellitus (IDD, type I diabetes) is an autoimmune disease caused by mononuclear cell infiltration of the pancreatic islets that leads to the selective destruction of insulin producing β cells (Gepts et al., 1965; Palmer et al., 1983). Progressive loss of islet beta cells results in insufficient insulin production, which causes clinical diabetes, characterized by hyperglycemia, ketonuria, glycosuria, polyuria, polydypsia, and weight loss. The inability to regulate blood glucose levels eventually results in death unless insulin is administered. In this disease, other pancreatic endocrine cells, such as somatostatin-producing cells, are not affected and autoantibodies to several pancreatic islet cells/antigens are detected before the onset of disease (Bottazzo et al., 1974).

Insulin-dependent diabetes in both humans and rodent models is a multifactorial disease which results from both genetic and environmental factors. Barnett et al. (1981) and Rotter et al. (1990) found the risk of disease for an identical twin of an individual with IDD to be more than 80 times the risk for the general population which strongly suggests a genetic contribution. However, the findings that among twins of patients with IDD only 33% subsequently develop diabetes and that 90% percent of patients with newly diagnosed IDD do not have an affected first-degree relative suggests that

environmental factors, such as diabetogenic virus, dietary constituents, or β cell-tropic toxins also influence the development of IDD (reviewed by Atkinson and Maclaren, 1994).

The Non-Obese Diabetic (NOD) Mouse

The non-obese diabetic (NOD) mouse is a well-established spontaneous animal model for IDD. Makino et al. (1980) developed the NOD mouse from a single diabetic female that spontaneously arose from a noninbred cataract-prone mouse strain. They established the NOD as a homozygous strain by inbreeding for over 20 generations. The NOD mouse shares many common immunological features with human IDD, such as the development of autoantibodies, insulitis, and ketosis-prone diabetes. NOD mice that develop diabetes die within a few weeks of onset unless they receive insulin therapy (Makino et al., 1980; 1981).

Insulin-dependent diabetes in the NOD mouse is influenced by many factors. Insulitis and diabetes susceptibility are under polygenic control and include both MHC and non-MHC related genes (Leiter, 1989; Wicker et al., 1996). Environmental factors also influence the incidence of disease and include dietary conditions, caging conditions, and specific pathogens such as murine hepatitis virus (Leiter et al., 1990).

The NOD, however, differs from humans in that diabetes incidence in females is markedly higher than in males. In females, the onset of diabetes is observed at approximately 8 weeks of age, with a cumulative incidence of 80% diabetes by 30 weeks of age. In contrast, in male NOD mice, the age of diabetes onset is similar to females, but the incidence by 30 weeks of age is only about 30% (Makino et al., 1981).

Polygenic Control of IDD Susceptibility

Inheritance of IDD and insulitis susceptibility are under polygenic control in the NOD mouse. Early studies showed that all crosses between the NOD and non-IDD susceptible control mouse strains resulted in F1 mice that did not develop insulitis and were free of diabetes suggesting the genetic control of diabetes is recessive (Ikegami et al., 1993).

Published studies have shown that defects in MHC molecules are associated with several autoimmune diseases (Erlich et al., 1993). MHC molecules play a crucial role in antigen presentation and T cell activation and may thus play a central role in T cell tolerance. In the thymus, MHC molecules present both foreign and self antigens to immature T cells. Through interaction via T cell receptors, T cells are chosen through positive and negative selection (Ashton-Richardt et al., 1994; Sebzda et al., 1994). In the periphery, the MHC also presents self antigen to self reactive T cells which causes activation induced cell death to maintain peripheral immune tolerance (Rocha et al., 1991; Zhang et al., 1992). The first IDD susceptibility locus (*Idd1*) to be identified was the NOD MHC II, designated H-2^{g7} (Hattori et al., 1986; Prochazka et al., 1987; Ikegami et al., 1988), a unique I-A molecule. Ikegami et al. (1988; 1993) reported that the CTS strain ($H2^{CTS}$) is a natural recombinant expressing the same class II gene as NOD (including a non-functional Eα gene) but different class I and III loci, and developed a NOD MHC congenic mouse expressing the CTS MHC. While NOD.H-2^{cts} congenic mice developed diabetes, the frequency was significantly less than in the NOD strain. These data support the hypothesis that the NOD I-A molecule is only partially responsible for

diabetes and that class I or III alleles also contribute to disease development. NOD and CTS differ at *Hsp70, Bat5*, and *Tnf*β in the class III region, and therefore, in addition to the class I loci, these loci are candidate genes for IDD. Thus, in addition to the genes encoding the I-A molecule, other genes within the MHC of the NOD may function together to create the effects of *Idd1.*

Although the NOD MHC is required for the development of diabetes, when the MHC of the NOD strain was expressed on the B10 or B6 genetic background, no insulitis or diabetes was observed (Ikegami and Makino., 1993; Wicker et al., 1993). The congenic strains, B10.H-2^{g7} and B6.H-2^{g7}, which express the NOD MHC, demonstrate that the MHC itself is not sufficient for developing pathology associated with diabetes and suggest that non-MHC genes also play a role in diabetes. Indeed, many non-MHC susceptibility loci have been localized to regions of different chromosomes. *Idd2,* located on chromosome 9 near the *Thy1* locus was the first of the many non-MHC linked *Idd* loci to be identified (41). Analysis of progeny from the (NOD x B10.H-2^{g7}) F1 x NOD BC1 generation also led to the mapping of other *Idd* susceptibility regions including *Idd3* (Ghosh et al., 1993; Todd et al., 1991; Wicker et al., 1994) and *Idd10* (Ghosh et al., 1993; Wicker et al., 1994) on chromosome 3, *Idd4* on chromosome 11 (Todd et al., 1991), *Idd5* on chromosome 1 (Cornall et al., 1991), *Idd6* on chromosome 6 (Ghosh et al., 1993), *Idd7* on chromosome 7 (Todd et al., 1991), *Idd8* on chromosome 14 (Todd et al., 1991), and *Idd9* on chromosome 4 (Rodrigues et al., 1994). Additionally, *Idd11* was mapped on chromosome 4, *Idd 12* on chromosome 14, *Idd 13* on chromosome 2, and *Idd14* on chromosome 13 (Wicker et al., 1995).

In addition, (NOD x B10.H-2^{g7}) F1 x NOD BC1 progenies were examined for pancreatic islet pathology to determine which *Idd* loci were linked to insulitis. It was found that *Idd3* and *Idd10* on chromosome 3 and *Idd5* on chromosome 1 showed significant linkage to insulitis (Ghosh et al., 1993; Todd et al., 1991; Wicker et al., 1994). Two more insulitis loci were later identified on chromosome 1 by a cross between NOD and C57BL/6 and NZW mice (Garchon et al., 1991).

With the discovery of these *Idd* loci, candidate genes have been suggested and examined for functional polymorphism. Since many loci have been localized to a large region of a particular chromosome which contains many genes, it makes candidate gene selection difficult. IL-2 has been proposed as a candidate gene for *Idd3* (Chesnut et al., 1993), Fc receptor for *Idd10* (Prins et al., 1993), and both Bcl-2 and *Lsh/Ity/Bcg* for *Idd5* (Cornall et al., 1991; Garchon et al., 1991). Prostaglandin synthase 2 (PGS-2) enzyme, the rate limiting enzyme for prostanoid metabolism, is responsible for the production of large quantities prostaglandins by MPs. The PGS-2 is located on chromosome 1 near *Idd5*. Due to the differential expression of the PGS-2 gene that I have observed in NOD in comparison to congenic and control mouse strains, and the correlation of this PGS-2 phenotype to the development of autoimmunity, I have proposed PGS-2 as a candidate susceptibility gene for *Idd5*.

Characterization of the Cellular Infiltration in NOD Insulitis

The development of insulin-dependent diabetes in NOD mice is preceded by the progressive accumulation of lymphocytes within the pancreatic islets of Langerhans (termed insulitis). Insulitis is first noted at 3 to 4 weeks of age, and virtually all female

and male NOD mice have insulitis by 2 months of age (Fujita et al., 1982). Histopathological examination of the pancreas of the NOD mouse reveals that the insulitis is initially confined to ducts and vascular areas followed by movement to the peri-islet areas, and progresses until the islet is surrounded by lymphocytes. By eight weeks in females, lymphocytes and monocytes penetrate into the islet itself and insulin-producing β cells are specifically destroyed while leaving delta and alpha cells intact (Wicker et al, 1986). Diabetes ensues after about 12 weeks when a large proportion of the islets have been destroyed.

Immunohistological studies show that macrophages, dendritic cells, B cells, and T cells are present in the pancreatic infiltrates with the majority of cells being Thy-1-positive T cells. Shimizu et al. (1987) have shown that among T cells, L3T4+ T helper cells are in greater numbers than the Lyt-2+ T cytotoxic cells. Miyazaki et al. (1985) found a predominance of T lymphocytes in the NOD islet from 3 to 13 weeks of age. Islets from 6 weeks old mice were characterized by a marked T-helper and T-cytotoxic cell infiltrate. Shimizu et al. (1987) found that the T lymphocytes were localized to the islets and proposed that they were responsible for beta cell destruction. B lymphocytes, though abundant, were observed more peripherally and islet cell specific antibodies were either not detected (Shimizu et al., 1987; Miyazaki et al., 1985) or detected late in the disease process, at 12-18 weeks (Kanazawa et al., 1984).

Jarpe et al. (1991) observed that islet infiltration of CD8+ T cells and MHC class II positive macrophages at 5-6 weeks of age, followed by a transient wave of CD4+ positive T cells and then by an influx of B cells and CD8+ T killer cells and proposed the

following model for the cellular time course of insulitis: antibody-dependent cellular cytoxicity and opsonization-phagocytosis at the early stage (5-6 weeks) followed by a transient wave of CD4+ T cells (6-8 weeks) followed by CD8+ T cell damage at the late stage (8-10 weeks).

T cell Activation Defects and Tolerance In NOD Mouse

Many studies suggest that T cells play a central role in the pathogenesis of β cell destruction in NOD mouse. A T cell role in disease has been suggested based on the observation that neonatal thymectomy prevents diabetes in NOD mice (Ogawa et al., 1985), and diabetes is transferred into irradiated young recipients by adoptive transfer of splenocytes from overtly diabetic NOD mice (Wicker et al., 1986). By using purified T cells and fractionated L3T4+ (CD4+) and Lyt-2+ (CD8+) T cells from diabetic NOD spleens, Bendelac et al. (1988) and Miller et al. (1988) demonstrated that both CD4+ and CD8+ T cells were required for transfer of disease while B cells were not. Shizuru et al. (1988) and Wang et al. (1991) further showed that diabetes in NOD mice is T cell dependent as it is prevented by injection of anti-CD4 antibodies.

T lymphocytes normally respond to a wide variety of foreign antigens and are tolerant to self antigens. Mechanisms for regulation of T cell self-tolerance can be found in both the thymus and in the periphery. The TCR of immature T cells differentiating within the thymus interacts with peptides presented by MHC gene products expressed on both hematopoietically derived APC and thymic epithelium (Sprent et al., 1987). The interaction of immature $CD^{3+/low}$CD4+CD8+ thymocytes with foreign peptides in the context of self MHC gene products induce positive selection of T cells. The T cells which

do not undergo positive selection are eliminated. To avoid the development of T cells bearing an autoreactive TCR, T cell precursors that recognize self-peptides presented in the context of self-MHC gene products are negatively selected by an activation induced cell death (AICD) process known as apoptosis (Finkel et al., 1989; Murphy et al., 1990). When mature peripheral T cells are fully activated, they are induced to follow one of three known paths: differentiation to effector cells (including suppressor cells), anergy, or activation induced cell death (Weber et al., 1990; Nigata et al., 1995; Dhein et al., 1995). AICD provides an antigen specific regulatory system dependent on the qualitative and quantitative signals provided by the interaction of antigen presenting cell (APC) and self-reactive T cell. Any defect intrinsic to APC or T cell affecting their interaction or subsequent T cell activation may possibly result in impairment of AICD. Impaired AICD could contribute to a common defect in autoimmunity: the accumulation and activation of self-reactive cells (Mountz et al., 1994; Thompson, 1995).

Many studies have demonstrated that treatment of the NOD mouse with high doses of glutamic acid decarboxylase (GAD) induces splenic T cell tolerance to this antigen and prevents diabetes (Tisch et al., 1993; Kaufman et al., 1993). GAD is the target of 64 kD reactive autoantibodies in both humans and NOD mice and is one of important islet cell autoantigens (Baekkeskov et al., 1990). Two forms of GAD (65 and 67 kD) exist (Bu et al., 1992). Both GAD 65 and 67 have been identified in islet cells and may play a role in the inhibition of somatostatin and glucagon secretion, as well as in the regulation of insulin secretion and proinsulin synthesis. Because treatment of the NOD mouse with GAD induces splenic T cell tolerance to this antigen, it suggests that T cell

tolerance is not complete in NOD mice but can be effectively established by activating autoimmune T cells with high doses of autoantigen. Studies have suggested that the activation of T cells by powerful stimuli such as high doses of antigen, superantigen, or by persistent encounters with self antigens on APCs leads to clone elimination *in vivo* (Web et al., 1990; MacDonald et al., 1991). Antigens that are processed and presented in an inefficient fashion can continue to stimulate T cells responses in the periphery, but would be unable to induce tolerance (Milich et al., 1989; Mamula, 1993). Similarly, the induction of activation-driven T cell death both in the periphery and during negative selection in the thymus, requires quantitatively more antigenic stimulation than is needed to induce positive selection or trigger effector T cell proliferative responses (Ucker et al., 1992; Sebzda et al., 1994).

These findings indicate that the threshold of T cell activation required to induce tolerance is much higher than that required to trigger an effector response. In addition, the stimulation of immunoregulatory T cell requires a more highly activated APC than is required to activate effector T cells (Ishikura et al., 1993). Thus, any genetic defect that compromises the differentiation or function of APCs could preferentially diminish the ability of these cells to present antigens in a manner quantitatively sufficient to induce tolerance and/or activate immunoregulatory T cells, without fully abrogating their ability to activate effector T cells.

Defects in both APCs and the T cell signaling pathway have been described to contribute to impaired T cell activation (De et al., 1994; Yokona et al., 1989; Ransanen et al., 1989; Serreze et al., 1993b; Rapoport et al., 1993). IL-2 production following APC -

T cell interaction appears to be of critical important for tolerance in general as mice that do not express IL-2, or the IL-2 receptor as a result of gene disruption develop severe generalized autoimmune disease (Sadlach et al., 1993; Willerford et al., 1995). Many studies suggest that there is impaired IL-2 production and activation of regulatory T cells in response to self antigen presenting cells and mitogens in both human and murine IDD (De et al., 1994; Yokona et al., 1989; Ransanen et al., 1989; Serreze et al., 1993b). This may be an important factor in impairing the development of tolerance in this disease.

The Role of the Macrophage in NOD Diabetes, APC Effect on Tolerance

The mechanism(s) by which the β cell is specifically destroyed is not known, but MPs in addition to T cells have been implicated. Jansen et al. (1994) demonstrated that prior to infiltration of T cells (3 week of age), MPs and DCs accumulate around the islets, with DCs remaining outside the islet, but MPs going on to penetrate the islet. Blocking the migration of MPs into the islet by treatment of NOD mice with CR3 (myelomomocytic adhesion promoting type-3 complement receptor) prevents intra-islet infiltration by both MPs and T cells and inhibits development of IDD (Hutchings et al., 1990). Furthermore, depletion of MPs by treatment of NOD mice with silica injections also blocks the development of insulitis and diabetes (Charlton et al., 1989; Lee et al., 1988). Macrophage accumulation in the pancreas may well have a major role in the processing and presentation of islet antigens to autoreactive T cells within the islet (Lee et al., 1988; Chariton et al., 1988). Additionally, activated MPs produce the monokines IL-1 and TNF-α that induce islet cell and MP production of NO, a potentially toxic metabolite to which β cells are highly sensitive (Cobet et al., 1995). Many other oxygen radical

species, produced by activated MPs, can also induce β cell death (Cobet et al., 1995; 1992).

NOD MPs differ from MPs of non-autoimmune control strains of mice in their development and function. Serreze et al. (1993a) found that NOD bone marrow cells proliferate poorly in response to the myeloid growth factor CSF-1 and generate fewer phenotypically mature (Mac-3^+) MPs than are generated from CSF-1 stimulated marrow from diabetes-resistant mouse strains. In addition, the finding that LPS-stimulated MPs from NOD mice secrete relatively little IL-1 (Jacob et al., 1990; Serreze et al., 1990) indicates that a large proportion of APCs in NOD mice may be incompletely differentiated, as MPs do not acquire the ability to secrete IL-1β until later stages of development. The reduced capacity to produce IL-1 may be of practicable importance as this cytokine may preferentially activate T helper type 2 (Th2) responses associated with disease protection in the NOD (Rabinovitch, 1994). Serreze et al. (1990) and Campbell et al. (1991) further demonstrated that the reduced ability of NOD mice to generate functionally mature MPs is associated with both aberrant regulation of RNA transcripts encoding the receptors for CSF-1 and IFN-γ, and a decreased ability to activate protein kinase C (PKC) second messenger activities coupled to these receptors. PKC activity is also reduced in NOD T cells which suggests a PKC induction defect in more than one cell lineage.

In addition to developmental defects, MHC associated defects intrinsic to MP and other APC may also contribute to the reduced capacity to active T cell. It has been found that NOD mice produce significant levels of IFN-γ (Campbell et al., 1991) and that MHC

class I, but not MHC class II, expression is aberrantly down-regulated in a cell-specific manner on NOD MPs, but not on pancreatic β cells exposed to interferon-gamma (IFN-γ) for 6 days (Serreze et al., 1993b). In contrast, MHC class I expression is up-regulated normally in IFN-γ treated macrophages from diabetes resistant NOR mice (Serreze et al, 1993b). Serreze et al. (1993b) and Kay et al. (1991) suggest that aberrant down-regulation of MHC class I expression by IFN-γ in NOD derived APC may impair the ability of these cells to mediate negative selection of autoreactive CD8+ T cells, which are then efficiently targeted to pancreatic β cells that express high levels of MHC class I.

The unique H-2^{g7} MHC molecule also plays a central role in T cell activation and murine IDD. IDD rarely develops in congenic stocks of NOD that express heterozygous MHC haplotypes from other strains and those immunotolerogenic defects most readily occur when the H-2^{g7} is homozygous (Wicker et al., 1992; Prochazka et al., 1989). The effect of the H-2^{g7} MHC molecule maybe due to its instability and decreased efficiency in binding and presenting self antigens as recently reported (Carrasco-Marin et al., 1996).

The finding that NON mice congenic for the H-2^{g7} haplotype (NOD.H-2^{g7}) are diabetes resistant (Ucker et al., 1992) indicates that APC expression of the diabetogenic MHC haplotype is insufficient for development of disease, and that the pathogenesis in NOD mice is dependent on synergistic interaction between diabetes susceptibility genes both inside and outside of the MHC.

The other defects in NOD MPs, in combination with the diabetogenic H-2^{g7}, may perturb MP processing and/or presentation of β cell autoantigens in such a way that their

ability to mediate negative selection of autoreactive T cells in thymus and periphery is impaired while retaining their ability to active "low level" effector functions.

The Impact of Prostaglandin E-2 on APC, T cell, and AICD

Prostaglandins, especially PGE-2, exert some very important effects during an immune response. PGE-2 produced early during the activation of MPs has proinflammatory effects such as vasodilatation, increasing vascular permeability, and under specific conditions stimulates MP cytoxicity. PGs also have a profound effect on monokine expression following the activation of MPs (i.e., after LPS stimulation). They readily suppress IL-1 and TNF-α expression while up-regulating IL-10 production through their effects mediated by cAMP on the IL-10 promoter (Meisel et al., 1996). PGE-2 also affects T cell cytokine expression and promotes Th2-like cytokine secretion profiles in murine and human CD4+ T cells by inhibiting Th1-associated cytokines IL-2 and IFN-γ and up-regulating the production of the Th2-associated cytokines IL-4 and IL-5 in a dose-dependent manner (Snijidewint et al., 1993).

Many studies have shown that PGE-2 modulates immune responses by markedly inhibiting T cell activation events including IL-2 and IL-2 receptor expression (Goetzl et al., 1995; Lee et al., 1993). The effects of PGE-2 are mediated by up-regulating the intracellular second messenger, cAMP, which binds to its intracellular receptor protein kinase A (PKA). As with other agents that increase cAMP (histamine, adenosine, forskolin, and cholera toxin), the mechanism by which PGE-2 activates PKA involves the binding of cAMP to the inactive tetrameric holoenzyme and its dissociation into two regulatory and two catalytic subunits (Krammer, 1988). This kinase-mediated activation

leads to the inhibition of IL-2R expression and IL-2 production (both mediated by a decrease in IL-2 nuclear transcription and IL-2 mRNA stability) (Anastassiou et al., 1992).

Activation induced cell death is an important mechanism for peripheral T cell tolerance (Ashton-Richardt et al., 1994; Sebzda et al., 1994; Rocha et al., 1991; Zhang et al., 1992). After lymphocytes mature and leave the thymus, they are functionally competent, e.g., capable of responding to antigenic stimulation by proliferating and differentiating into effector cells. Among the progeny of antigen-stimulated lymphocytes, only a small fraction develop into functional effector and memory cells. The majority probably die by apoptosis (Rocha et al., 1991; Zhang et al., 1992). The process of activation-induced cell death may be enhanced by the exposure of antigen stimulated lymphocytes to growth factors, such as IL-2 in the case of T cells (Klas et al., 1993). In mice, administration of large doses of anti-T cell receptor antibodies or superantigens, such as staphylococcal endotoxins, results in the deletion of T cells that express antigen receptors which specifically bind these antibodies or superantigens (Trauth et al., 1989; Kawabe et al., 1991). This is due to activation induced apoptosis, and may be an exaggerated version of the phenomenon that normally occurs in clones of antigen-specific lymphocytes that encounter self antigen. Activation induced cell death in mature lymphocytes, therefore is a homeostatic mechanism that functions to regulate the number of antigen-stimulated clones and also serves a highly protective function in the case of autoantigens. Indeed, impaired AICD, as in *lpr/lpr* mice, contributes strongly to the development of autoimmunity (Wu et al., 1994). It has been suggested that T cell receptor

(TCR) stimulation of resting mature peripheral T cells causes them to undergo activation, but not AICD, whereas TCR stimulation of cycling cells causes a significant number of lymphocytes to die. Some critical events for AICD include TCR activation, lymphokine mediated cell cycle progression (IL-2 production), and TCR re-engagement (Guery et al., 1995; Stockinger et al., 1992). Several studies have suggested that AICD can be markedly inhibited by anti-IL-2 antibodies and by agents that block the cell cycle, i.e., cAMP (Boehme et al., 1993; Critchfield et al., 1995).

Yokono et al. (1989) demonstrated that aberrant PG production from NOD MPs contributes to the suppression of T cell activation and IL-2 production in Con-A stimulated spleen cells in NOD mice. Preliminary studies in our lab have also shown that MPs are responsible for the suppression of T cell activation in NOD syngeneic mixed lymphocyte response (SMLR) and that PGs mediated this defect.

Because of PG effects on T cell activation, these molecules may profoundly affect AICD. Several studies have shown that PGE-2 markedly impairs AICD of cycling mature T cells through activation of adenylate cyclase and generation of cAMP (Goetzl et al., 1995; Ucker et al., 1994). The quantitative signal given by the interaction of IL-2 and IL-2R has been shown to be decisive in cell activation, proliferation, AICD, and immune tolerance (Sadlack et al., 1993; Willerford et al., 1995). PGE-2 is a potent inhibitor of IL-2 and IL-2 receptor expression and also induces high levels of cAMP and blocks cell cycle in T cells. The mechanism by which cAMP protects T cells from apoptosis may involve its inhibition of Ras-dependent activation of the signal transmission pathway leading from Raf to mitogen-activated protein kinases (Cook et al., 1993). This inhibitory

mechanism is postulated to require protein kinase A. In addition, PGE-2 appears to play a regulatory role in the function of ceramide, a main AICD signal transducer (Jarvis et al., 1994; Hannum et al., 1994). Ceramide in turn promotes the production of PGE-2 though induction of PGS-2, thus promoting its own regulation (Hannum et al., 1994).

An established *in vivo* experimental model of AICD is the immunization of mice with the bacterial superantigen, *Staphylococcus aureus enterotoxin B* (SEB) (MacDonald et al., 1991; Kawabe et al., 1991). In this experimental paradigm, SEB immunization of BALB/c mice initially leads to the expansion of SEB reactive T cells bearing the Vβ8+ T cell receptor (Vβ8+) within 48 hours of immunization. By 10 days post immunization, however, Vβ8+ T cells are reduced secondary to cell death. In contrast, control T cells that are not activated by SEB, i.e., Vβ6+ TCR bearing T cells do not expand nor are they deleted in SEB immunized mice.

Macrophage Activation and PGS-2 Expression

Cells of the mononuclear phagocyte system originate in the bone marrow, and after maturation and subsequent activation achieve varied morphological forms and functions. The first cell type that enters the peripheral blood after leaving the marrow is incompletely differentiated and is known as the monocyte. Once the monocyte migrates into tissues, these cells undergo further maturation and become tissue specific macrophages. In tissues, their functions are strongly down-regulated by a variety of suppressive factors normally present. They therefore remain in a quiescent state until stimulated or challenged with a wide variety of agents such as lympokines from lymphocytes, tumor cells, bacteria, foreign particles, or environmental toxins. Once

activated, MPs become able to produce TNF-α, IL-1, oxygen radicals, NO and various immunoregulatory factors that include components of the complement system and arachidonic acid metabolites such as prostaglandins (PGs) (Meisel et al., 1996). Following LPS activation of MPs, there is a defined order of monokine mRNA and protein expression with TNF-α and IL-1 expression preceding the expression of PGS-2, and IL-10 following PGS-2 expression (Meisel et al., 1996). The order of expression for these monokines is critical as there are many complex regulatory interactions amongst them. It has been found that TNF-α and IL-1 up regulate PGS-2 and IL-10 expression. After PGS-2 is expressed, PGE-2 production potently suppresses TNF-α and IL-1 expression while up-regulate IL-10 production. IL-10, in turn, suppresses the expression of TNF-α, IL-1 and PGS-2 (Mertz et al., 1994).

The activation of MPs, however, is not a singular process. Activation consists of quantitative alterations in the expression of various gene products (proteins) that endow the activated MPs with the capacity to perform some functions that can not be performed by the resting MP such as tumor killing (Mackey et al., 1993; 1987). Two of the most extensively studied marker proteins for MP activation in tumor cell killing are the proteins p47b and p71/73 (Mackey et al., 1993; 1987). Unstimulated MPs do not express either marker, whereas expression of p47b alone is evidence that a MP has become primed by interferon, and simultaneous expression of both p47b and the p71/73 complex correlate with full activation and tumor cell killing. Recently, p71/73 has been identified as an inducible prostaglandin endoperoxide synthase (cyclooxygenase 2 or PGS-2) (Xie et al., 1993).

Prostaglandin endoperoxide synthase is the rate limiting enzyme in prostanoid metabolism. The biosynthetic pathways for PGs or leukotrienes include three distinct stages: (1) release of arachidonic acid from membrane phospholipids which provides substrate, (2) metabolization of free arachdonic acid (AA) to leukotrienes by lipooxygenase or oxygenation by cyclooxygenase to yield PGH, a prostaglandin endoperoxide that serves as precursor for other prostaglandins, and (3) depending on the enzymes present in the cell, conversion of PGH to different prostanoids (including PGE-2, PGD-2, prostacyclin, and thromboxane). The biosynthetic pathways are summarized below in Figure 1.

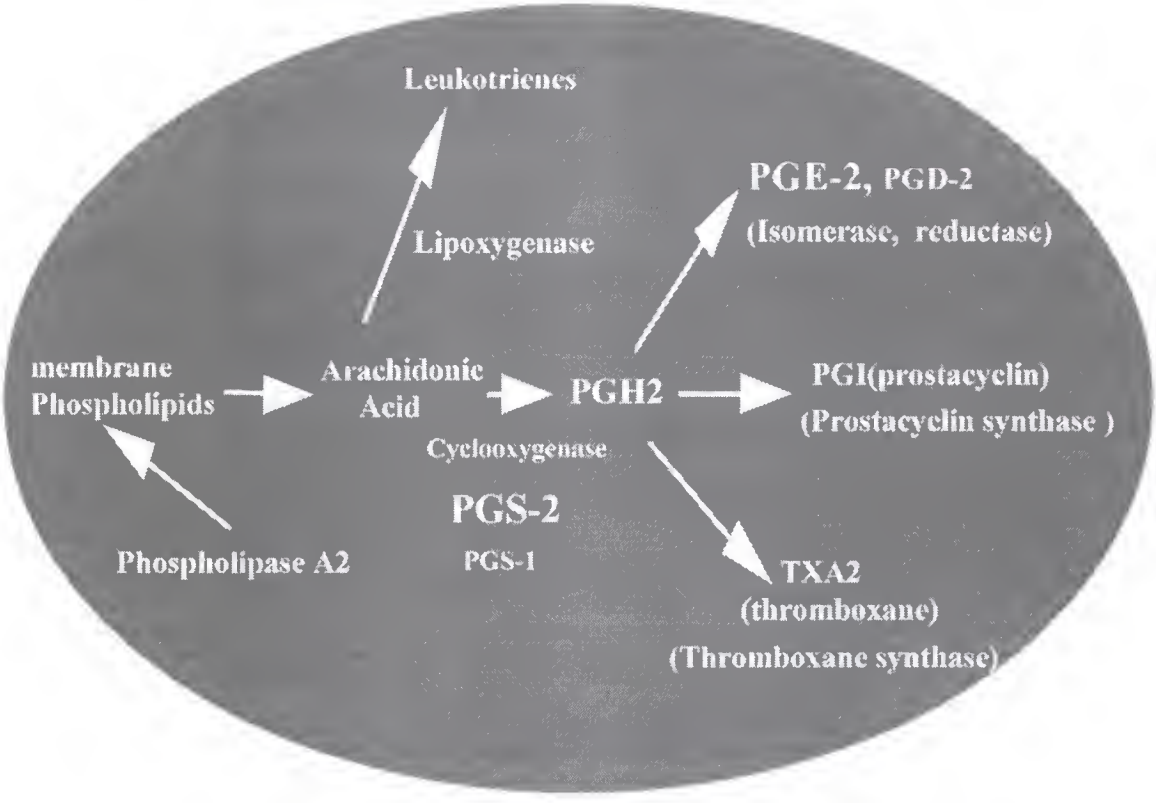


Figure 1. Prostanoid Metabolism

AA release involves either the action of phospholipase A2 (PLA2) on phosphatidylcholine or phosphatidylethanolamine, yielding arachidonate, or the action of a phospholipase C on phosphatidylinositol, yielding diacylglycerol, which in turn undergoes cleavage to give free arachidonate. There are two isoforms of PLA2, secretory PLA2 (sPLA2) and cytosolic PLA2 (cPLA2). They have similar functions, but effect AA metabolism at different locations (Glaser et al., 1995). PLA2 activation and expression occur as a result of tissue-specific stimuli by hormones such as bradykinin, epinephrine, or proteases such as thrombin (Balsinde et al., 1990). Release of AA can also occur if membranes are perturbed.

Mouse and human cells express two prostaglandin synthases, PGS-1 and PGS-2. PGS-1 is constitutively expressed, responsible for the production of low levels of prostanoids, and serves physiological housekeeping functions. PGS-2 is an inducible enzyme responsible for the production of large quantities of PGs (Xie et al., 1993). In contrast to PGS-1, PGS-2 mRNA and protein are not expressed in resting MPs but can be induced by mitogens, LPS, and cytokines such as IL-1, TNF-α (Rzymkiewicz et al., 1994; Tordiman et al., 1995).

Constitutive PGS-1 Expression and PGS-2 Inducibility

Both PGS genes are single copy genes. They are located on different chromosomes. PGS-1 maps to chromosome 2 in the mouse (Xie et al., 1993) and 9 in the human (Kosaka et al., 1994). PGS-2 maps to chromosome 1 in both species (Xie et al., 1993; Kosaka et al., 1994; Jones et al., 1993). Both human and murine PGS-1 are ~22 kb in length and contain 11 exons and 10 introns with the exon-intron structure conserved

between humans and mice. The murine PGS-1 cDNA encodes a 602-aa polypeptide, containing a 26-aa signal peptide and its mRNA is about 2.7 kb. PGS-1 is localized to the membrane of endoplasmic reticulum (ER). It is constitutively expressed in almost all tissues and cells and plays an important role in maintaining normal vascular, gastric, and renal homeostatic functions (Wang et al., 1993).

In contrast to PGS-1, the PGS-2 gene is ~8 kb in the length and contains 10 exons and 9 introns (Xie et al., 1991; Kujubu et al., 1991; 1993). PGS-2 cDNA has been cloned from human, rat, mouse, and avian sources. Comparison of PGS-2 sequences among these species shows about 80% identity and about 60% homology with PGS-1. All PGS-2 cDNAs encode a 604-aa polypeptide with four potential N-glycosylation sites. The signal peptide (17 aa) of the PGS-2 protein is shorter than the PGS-1 signal peptide. Near the C-terminus of PGS-2, there is an 18-aa insert which is absent in PGS-1. This protein sequence has been used to generate PGS-2 specific antibody that uniquely binds to PGS-2.

The single best characterized distinction between PGS-1 and PGS-2 is their differential regulation of expression. PGS-1 is expressed constitutively in almost all tissues, whereas PGS-2 is selectively expressed, primarily in macrophages, endothelial cells, fibroblasts, and smooth muscle cells, but only in response to activating agents such as cytokines, growth factors, hormones, and tumor promoters. The PGS-2 mRNA expressed in these cells is 4.0-4.5 kb, which is longer than that of PGS-1 (2.7 kb). In contrast to PGS-1, which localizes to endoplasmic reticulum (ER), PGS-2 preferentially

localizes to the nuclear envelope (Morita et al., 1995) and suggests that prostanoids formed via PGS-2 may directly function within the nucleus.

Constitutive PGS-1 expression suggests that cells using PGS-1 to produce prostaglandins involves a rapid response to stimulation by circulating hormones. Because of the time lag required for PGS-2 induction in a cell or tissue, this enzyme is available to produce prostaglandins only after activation following specific physiological events, such as inflammation, mitogenesis, and ovulation (Hedin et al., 1987; Wong et al., 1989). Recent evidence suggests that the PGS-1 and -2 produce prostaglandins through separate pathways and use different phospholipases that are coupled to different signaling pathways (Reddy et al., 1994; Murakami et al., 1994).

The regulation of PGS-2 has been extensively investigated by examining its mRNA and protein expression following stimulation in cultured fibroblasts, endothelial cells, and in purified macrophages or MP cell lines. PGS-2 expression can be induced by phorbol esters such as phorbol-12-myristate-13-acetate (PMA) (Rysecket al., 1992), TNF-α, IL-1 (O'Banion et al., 1992; Ristimaki et al., 1994; Rzymkiewicz et al., 1994), serum (DeWitt et al., 1993), growth factors, and LPS (Habib et al., 1993; Tordiman et al., 1995). Induction of PGS-2 by these stimulators is inhibited by cycloheximide which is in keeping with the concept that PGS-2 is a primary response gene. The magnitude of induction can reach 50 fold over the basal level (DeWitt et al., 1993).

The induction of PGS-2 is rapid. PGS-2 mRNA expression is induced ~30 min after stimulation, peaks at 1 hr, and returns to basal level at 4 hr (DeWitt et al., 1993). PGS-2 mRNA level, which peaks 1 hr following the addition of serum, declines by more

than 50% by 3 hr, suggesting that half-life ($t_{1/2}$) of PGS-2 mRNA is 2 h or less. PGS-2 protein levels begin to increase immediately following induction of PGS-2 mRNA and are detectable 1 hr post induction. Protein levels peak at 2 to 4 hr after induction and return to near baseline levels by 6 hr (DeWitt et al., 1993).

A class of agents, the non-steroid anti-inflammatory drugs (NSAIDs), inhibit the enzymatic activity of PGS. Most PGS inhibitors such as indomethacin are non-selective and inhibit both PGS-1 and PGS-2 (Meade et al., 1993). New NSAID drugs are being developed, such as NS-398, which are selectively inhibits PGS-2 enzyme activity (Futaki et al., 1994), and have an important therapeutic application in inflammatory diseases. In addition, other physiological inhibitors of PGS-2 exist, such as glucocorticoids which also selectively inhibit PGS-2 but not PGS-1 (DeWitt et al., 1993). Anti-inflammatory cytokines such as IL-10, TGF-β also inhibit PGS-2 expression (Mertz et al., 1994).

Regulation of PGS-2 Expression

Nuclear run-off experiments in murine NIH3T3 cells indicated that serum increases the rate of PGS-2 mRNA synthesis (DeWitt et al., 1993; Evett et al., 1993). Increases in mRNA synthesis in fibroblasts were noted 12-30 min after the addition of fetal calf serum. The magnitude of the increase paralleled that of steady-state mRNA levels. Induction of PGS-2 protein by mitogenic growth factors, cytokines, and hormones has been attributed to increased transcription.

Sequence analysis of the untranslated 5'-flanking region of PGS-2 and PGS-1 has begun to shed light on the regulation of PGS-2 transcription. There are several consensus sequences for transcriptional activation on the 5'-flanking region of the human and

chicken PGS-2 gene (Figure 2). The PGS-2 gene has a canonic TATA box 30 bp upstream from the transcription start site (Tazawa et al., 1994; Xie et al., 1993). It contains several putative regulatory elements in the 280 bp of the 5'-flanking region: cyclic AMP response element, IL-6 response element (NF-IL6), C/EBP, AP-2, nuclear factor-κβ (NF-κβ), and SP-1 sites. Further upstream are putative PEA-3, GATA-1, NF-κβ, and NF-IL6 binding sites. The PGS-2 gene has features of a primary response gene and is expected to be inducible by phorbol ester, cAMP, and a number of cytokines and growth factors.

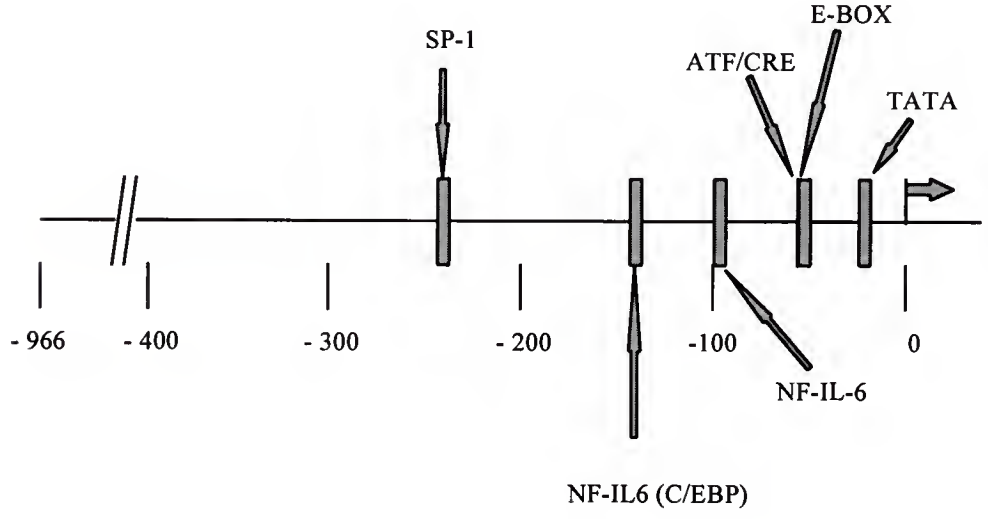


Figure 2. The Presumptive *cis*-acting regulatory sequences present in the PGS-2 promoter.

PGS-2 expression is also regulated at post-transcriptional levels (Evett et al., 1993; Ristimaki et al., 1994). PGS-2 mRNA is unstable compared with PGS-1 mRNA, a feature predicted from the presence of multiple RNA instability sequences (AUUUA) in

its 3'-untranslated region. PGS-2 mRNA is translated as soon as it is synthesized; therefore, the short mRNA half-life limits PGS-2 production post-transcriptionally (DeWitt et al., 1993).

PGS-2 expression is regulated at the protein level as well. PGS-2 protein is much less stable than PGS-1 in fibroblasts, indicating a post-translational regulatory mechanism that limits PGS-2 protein levels in fibroblasts (DeWitt et al., 1993). What accounts for the different protein stability of PGS-1 and PGS-2 is not known, but increased protein turnover of PGS-2 may be mediated via the carboxyl-terminal protein sequences that are unique to PGS-2.

Sex Hormone, The Immune Response and Autoimmunity

Human autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and multiple sclerosis are characterized by a disproportionate effect on women with a marked increase in disease post pubertal peroid (Cutolo et al., 1988; 1986). Animal models of human autoimmune disease similarly reflect a female bias, i.e. in NZB/W mice, a model of SLE, and in the NOD mouse. The higher incidence of disease in females suggests that higher concentrations of sex steroids in females, or their hormonal cycle may strongly influence autoimmune immunopathogenesis. Conversely, male sex steroids appear to inhibit expression of autoimmune disease (Fox, 1992; Homo-Delarch et al., 1991; Wilder, 1995).

In the NOD mouse, there is a marked difference in the incidence and the onset of diabetes between male and female mice with female NOD mice developing diabetes at three times the rate of their male counterparts. The influence of sex steroid hormones has

been demonstrated by exacerbation of disease in orchiectomized or estrogen treated NOD male mice, and a reduction in insulitis and diabetes incidence following oopherectomy or treatment with androgens in female mice (Fox, 1992; Hawkins et al., 1993; Fitzpatrick et al., 1991). Although experimental studies both *in vivo* and *in vitro* have confirmed the influence of sex hormones on immunoreactivity, the mechanisms of their influence have yet to be fully elucidated.

Studies have shown that hormone receptors are present in many cells involved in the immune response and the concentration of hormone levels play a major role in the regulation of the immune response. Evidence has accumulated to support the concept that female sex steroid hormones have a regulatory role in macrophage gene expression and effector function (Zhang et al., 1988; Wang et al., 1988). *In vivo* regulation may be either indirect or direct. For example, macrophages migrate into the estrogen-stimulated mouse uterus (Zhang et al., 1988), possibly as an indirect response to chemoattractive cytokines such as colony-stimulating factor-1 and granulocyte-macrophage colony-stimulating factor produced by estrogen-targeted uterine cells (Wang et al., 1988). However, *in vitro* studies show that estrogen and progesterone have direct effects as well. Sex hormones may inhibit or stimulate, in a dose-dependent manner, the Fc-mediated clearance of antibody-coated erythrocytes by guinea pig spleen macrophages (Schreiber et al., 1988) and may regulate expression of MHC class II (Iα) antigen and interleukin-1β (IL-1β) protein synthesis by mammalian monocytes, macrophages, and macrophage cell lines (Polan et al., 1989). The effects exerted by estrogen on MP IL-1 synthesis seem to be biphasic and dose-dependent. Polan et al. (1989) reported that a negative relationship has

been found between IL-1 mRNA levels and estrogen concentrations in cultured human peripheral monocytes and pelvic macrophages. In particular, IL-1 mRNA levels decreased by 80-90% as the measured estradiol concentrations increased from 10^{-9} to 10^{-5} M. Thus, low estrogen level (physiological, 10^{-7} M) stimulates both IL-1 mRNA levels and IL-1 protein secretion, whereas higher levels (pharmacological, 10^{-6} to 10^{-5} M) are inhibitory. Levels of circulating estradiol-17β (E2) and progesterone (P) in female mice during the estrus cycle and pregnancy has been reported to reach 60.0 pg/ml (~10^{-6} M) and 39.0 pg/ml (~10^{-7} M), respectively. In human placentas, P levels can vary from 0.5 ug/ml to 5.1 ug/ml. Recently, Miller et al. (1996) found that concentrations greater than 0.1 ug/ml of P inhibited iNOS gene activity and NO production, while E2 had no effect on iNOS gene activity and NO production in IFN-γ/LPS-stimulated macrophages.

It has been demonstrated by many investigators that prostaglandin E2, which is one of the major secretary products of monocytes, plays an important role in the immune response. El Attar et al. (1982) studied the effects of sex hormones on the production of PGE-2 using human gingival tissue and demonstrated that addition of estradiol or progesterone significantly enhanced the synthesis of PGE-2. They also reported that inflamed gingiva produced more PGE-2 than healthy gingiva and speculated that inflammatory cells in the tissue were responsible for the increased level of PGE-2. Miyaga et al. (1993) investigated the effect of sex hormones on the production of PGE-2 by LPS-stimulated human monocytes and showed that testosterone reduced the production of PGE-2, while progesterone enhanced it. Estradiol showed a bi-directional effect on PGE-2 production; that is, inhibitory at 0.4 ng/ml and stimulatory at 29 ng/ml.

Thus, the concentration of estradiol seems to be an important factor for this effect. They also found that the reduced PGE-2 production by monocytes treated with low amounts of estradiol was restored in the presence of high amounts of progesterone, while the enhancing effects of progesterone on PGE-2 production were reduced by addition of low amounts of estradiol. These results suggest that a balance in the combination of sex hormones modulates PGE-2 production by monocytes and macrophages in a female hormonal milieu.

The mechanism by which sex hormones affect MPs production of PGE-2 remains unclear. Landers et al. (1992) proposed the following general model for steroid action: Free steroids passively diffuse into all cells but are preferentially retained in target cells through the formation of a high affinity complex with the steroid-specific receptor. Binding of the steroid results in an "activation" of the receptor molecule that appears to involve conformational and post-translational changes in the receptor itself, as well as changes in the protein-protein associations in the receptor complex. Finally, the activated complex (steroid-receptor) binds with high affinity to specific DNA sequences, termed "steroid response elements" (SRE). The steroid response element acts as a transcription factors, modulating the rate of transcription of steroid-responsive genes or altering the post-transcriptional steps which, in turn, results in a change in the steady state levels of specific messenger RNAs (mRNAs) (Landers et al., 1992).

Regulation of Lymphocyte Functions by Nitric Oxide

Nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to L-citrulline and nitric oxide (NO) (Marletta et al., 1993). NO possesses diverse activities and is

considered to play an important role in many physiological functions and pathological conditions. Notably, it is a potent vascular mediator which maintains vascular homeostasis via its actions on platelets and vascular tone. It also acts as a neurotransmitter for central and peripheral nervous system function and as a nonspecific immune modulator involved in controlling the invasion of microorganisms and tumors (Moncada et al., 1991). Synthesis of this ubiquitous, diffusible gas in many tissues is catalyzed by isoforms of NO synthase (Marletta et al., 1994). As with PGS, two major types of NOS have been identified (Marletta et al., 1994). One is the calcium-dependent form, which is present constitutively in a variety of tissue and produces the physiological concentration of NO needed for 'house-keeping'. Another one is not consititutively expressed, but can be induced in a number of cell types, including macrophages, hepatocytes, neutrophils, muscle, and endothelium, via a variety of immunological stimuli such as interferon-γ, tumor necrosis factor, and LPS. Once induced, these cells produce large amounts of NO, which may be cytotoxic. Thus, apart from maintaining normal physiological function, NO is required in large amounts to combat infectious organism and tumors. Production of excessive amounts of NO will, however, lead to a different range of pathological outcomes and important pathologies. Therefore, the expression of inducible NOS (iNOS) is necessarily under tight regulation. Lipopolysaccharide and IFN-γ response elements have been located in the promoter region of iNOS gene (Marletta, 1994). A number of cytokines are able to inhibit the expression of iNOS by murine macrophages. These include TGF-β (Ding et al., 1990), IL-4 (Liew et al., 1991), and IL-10 (Bogdan et al., 1991). Glucocorticoids can also

suppress iNOS expression. Activation of PTK and PKC, in turn, activates iNOS, while inhibition of these kinases blocks NO production (Marletta., 1994; Bogdan et al., 1991).

NO produced by MPs plays an important role in affecting and modulating the immune response. NO, like PG, is also a potent inhibitor of T cell activation (Liew et al, 1991). Hoffman et al. (1990) first reported that NOS activity was detected during phytohaemagglutinin (PHA)-stimulated proliferation of rat spleen cells and also in a mixed lymphocyte response, and that the addition of NOS inhibitor, N-monomethy-L-arginine (NMMA), to cultures suppressed NOS activity and allowed a robust proliferative response to occur. These findings have since been extended to both *in vitro* and *in vivo* murine models, indicating that NO inhibits the proliferation of T cells. Taylor-Robinson et al. (1994) demonstrated that the inhibition of proliferation of Th1 cells by NO can be reversed by the addition of exogenous IL-2, suggesting that NO inhibits the expansion of Th1 cells by blocking the secretion of IL-2, which is an autocrine mediator of T cell expansion. Another way NO can influence T-cell function is through its modulation of antigen presentation by down-regulation of the expression of class II MHC molecules on antigen-presenting cells (Sicher et al., 1994). At present, the mechanism of such down regulation is unknown. When a panel of cloned T cells specific for malaria antigens was examined, it appeared that only Th1 cells could be induced to produce NO, whereas Th2 cells could not (Taylor-Robinson et al., 1994). This suggests low levels of NO produced by Th1 together with the NO produced by activated macrophages, may inhibit Th1 proliferation by blocking the synthesis of IL-2. It is likely that NO at a physiological concentration (provided constitutively) is required for proliferation of T cells (and indeed

for that of other cells). At the higher concentrations, however, NO inhibits cellular proliferation.

NO plays an important role in the immunopathogenesis of murine autoimmune diseases including IDD, arthritis, and SLE. Treatment of SLE prone mice with the NO inhibitors, such as the arginine analogue NMMA or aminoguandine (AG), reduces clinical autoimmune disease (Weinberg et al., 1994). Increased NO production and iNOS expression has been shown in MRL-*lpr/lpr* mouse (Wu et al., 1994). Nitric oxide also contributes directly to the pathogenesis of autoimmune disease by causing an increase in vascular permeability. Cell-derived NO, through interaction with superoxides forms peroxynitrite may spontaneously produce hydroxyl radicals. These radicals are highly reactive and readily cause cell and tissue injury and destruction. Evidence has shown that cytokines, like IL-1, released in islets by nonendocrine cells, most probably MPs can induce the expression of iNOS by β cells and inhibit their insulin secretion (Corbet et al., 1995).

iNOS and PGS-2 Crosstalk

In several physiological and pathological conditions, NO and prostanoids work synergistically. A recent report indicates that the inducible forms of PGS (PGS-2) and NOS (iNOS) are concurrently induced in inflammatory tissues in experimental animals (Vane et al., 1994). Although the inducing agents were not investigated in the experimental model, inflammatory cytokines, most notably IL-1β, have been shown to induce PGS-2 and iNOS in cultured cells.

PGS-2 and iNOS may also interact via their metabolites. Several reports have shown that NO increases prostanoid synthesis (Salvemini et al., 1993; Franchi et al., 1994). Although one study suggested that NO directly activates PGS-2 enzyme activity (Salvemini et al., 1993), further work is needed to determine whether the effect of NO is mediated by its direct activation of PGS-2 or via an intermediate step (Marshall et al., 1987). Conversely, PGS-2 metabolites have been reported to influence iNOS induction. A recent report showed that PGE-2 and iloprost, a PGI-2 stable analog, at micromolar concentrations, suppressed iNOS induction in an murine macrophage cell line, J774, whereas PGF-2a and lipoxygenase metabolites had no effect (Morotta et al., 1992).

In summary, there exists an intricate relationship between PGS-2 and iNOS under physiological and pathological conditions. Synergistic interactions of these two enzymes and their metabolites may play an important role in modulating the immune response and inflammation.

Rationale for these Studies

In NOD mice, both T cells and macrophages (MPs) play an important role in the immunopathogenesis of diabetes. Several studies have suggested that antigen presenting cells from autoimmune humans and animals including NOD mice, are defective in their capacity to active T cells (Serreze et al., 1988; Yokono et al., 1989). The cellular and molecular basis for this defect, however, is largely unknown. Studies from our lab have suggested a cellular basis for this defect, as NOD MPs, unlike those of control strains, suppress T cell activation through the production of prostaglandin and nitric oxide. Furthermore, I have established that NOD MPs display an "activation" phenotype, as

resident peritoneal MPs from NOD, unlike MPs from control MPs spontaneously and constitutively express an early response gene, prostaglandin synthase II (PGS-2).

PGS-2 is the rate limiting enzyme in prostanoid metabolism and responsible for the production of large quantities of PGs, therefore, I hypothesized that constitutive expression of PGS-2 may result in enhanced production of PGE-2 which may disturb peripheral tolerance in NOD mice through impair activation induced cell death.

Activation induced cell death (AICD) is an important mechanism for peripheral T cell tolerance. Indeed, impaired AICD, as in the *lpr/lpr* mouse, contributes strongly to the development of autoimmunity. Several studies have suggest that AICD can be markedly inhibited by anti-IL-2 antibodies and by agents that block the cell cycle and IL-2 production/signaling, i.e., cAMP (Boehme et al., 1993; Critchfield et al., 1995). Of interest, PGE-2 is a potent inhibitor of IL-2 and IL-2 receptor expression and can block the cell cycle through its activation of adenylate cyclase and generation of cAMP (Goetzl et al., 1995; Ucker et al., 1994). The potentially enhanced PGE-2 production in NOD MPs may therefore impair AICD in NOD mice and contribute to the pathogenesis of diabetes.

The overall objective of this proposal is to establish the role of PGS-2 and PGs in the immunopathogenesis of NOD diabetes. The specific aims include: 1) understanding the mechanism of the aberrant PGS-2 expression, 2) determination of the correlation between PGS-2 phenotype and antoimmunity phenotype and evaluating the role of PGS-2 as a candidate gene for *Idd5*, and 3) establishing a potential mechanism and the role of PGS-2 expression in impaired peripheral tolerance in NOD mice.

To fulfill these aims, I investigated 1) PGS-2 expression in the autoimmunity environment, 2) prostaglandin production in NOD MPs, 3) possible sequence variations of cis-elements of NOD PGS-2 promoter, 4) the influence of sexual hormones on PGS-2 expression, and 5) monokines regulation of NOD PGS-2 expression.

I also analyzed PGS-2 phenotype of different chromosome 1 congenic mice and compared PGS-2 expression with the autoimmune phenotype in these congenic mice which help me elucidate the role of PGS-2 as a candidate gene for *Idd5*.

To further investigate the role of PGS-2 in the pathogenesis of diabetes, we used drugs to block PGS-2 activity and examined the effect of drug treatment on the onset and incidence of diabetes.

Finally, I established a potential mechanism for PGS-2 expression in the impairment of immune tolerance by using *Staphyloccus aureus enterotoxin B* (SEB) immunization as a model to examine AICD in NOD mice.

From these studies, I elucidated the role of PGS-2 expression in NOD diabetes and have derived a better understanding of the pathogenesis of this autoimmune disease.

CHAPTER 2
MATERIALS AND METHODS

Animals

Female and male NOD mice were purchased from the Taconic Laboratory (Germantown, NY) and BALB/c and B57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) for experiments conducted in the first and the second years. The NOD scid/scid mice, B6.NODC1 congenic mice, and NOD.B10C1, NOD.B10C3, NOD.B10C4 congenic mice were gifts from our collaborators: Dr. Mark Atkinson (University of Florida, Gainesville, FL), Dr. Edward Wakeland (University of Florida, Gainesville, FL), and Dr. Linder Wicker (Merck Laboratory, Rahway, NJ). NOD, BALB/c, C57BL/6 and NOD.H-2^b congenic mice used for experiments in this year were bred and maintained in the animal facility of the Department of Pathology and Laboratory Medicine at the University of Florida, Gainesville. All the mice, except B6.NODC1 congenic strain, were housed in SPF conditions. Estrus phase of female mice was determined by vaginal cytology.

For the first set of PGS-2 inhibition experiments, NOD female mice were treated from 8 weeks to 32 weeks of age with indomethacin (3 ug/ml) and aminoguanidine (0.1%) in their drinking water. In a second study, NOD female mice were treated from 4 weeks to 22 weeks of age with indomethacin (20 ug/ml) in their drinking water. Water was changed every week and the drugs freshly added at each change. Mice were housed

in SPF conditions during the entire experimental peroid. Development of diabetes was monitored weekly by a urine glucose test and confirmed by tail vain blood glucose (250 mg/dl glucose was used as indication of diabetes).

For the SEB immunization, 6-8 week old female mice were given an intraperitoneal injection of buffed saline or SEB (12.5 ug or 50 ug). Spleen cells were examined at day 2 and day 10 after immunization.

Antibodies

PGS-2 specific antisera was produced in Dr. Harvey Herschman's lab. Antibodies for the FACS analysis including FITC labeled anti-mouse Vβ 6 TCR and Vβ 8.1, 8.2 TCR, PE labeled anti-mouse CD4 and relevant isotype control antibodies were purchased from Pharmingen Bio. Inc. (San Diego, CA).

Peritoneal Cell Preparation and Culture

Mice were sacrificed by cervical dislocation and cells were obtained by washing the peritoneal cavity with 5 ml of cold 10% endotoxin-free FBS-supplemented RPMI 1640. For mRNA determination, adherent resident peritoneal MPs were isolated by incubating $1x10^5$ MPs with RPMI 1640 (GIBCO, Grand Island, NY) which containing 2 mM glutamine, 100 u/ml penicillin, and 100 ug/ml streptomycin, plus heat treated 10% FBS (HyClone Laboratories, Logan, UT) for 2-3 hr in 96 well plates (Costar Corporation, Cambridge, MA) at 37^0C in a 5% CO_2 atmosphere. Cells were washed three times with same media and cultured for 16 hr alone or with stimulation: LPS (10 ug/ml) or hormones at physiological concentrations. Hormones used were: estradiol (10^{-6} M), progesterone (10^{-7} M), testosterone (10^{-7} M), dehydroepiandrosterone (DHEA, 10^{-7} M),

and dehydroepiandrosterone sulfate (DHEAS, 10^{-7} M). MPs were released from plate by adding 50 ul ice cold EDTA-PBS buffer (0.02% EDTA) to the MPs and incubating for 15 min on the ice. For castration experiments, NOD males were castrated at 8 weeks of age. Four weeks after castration, MPs were collected as described above, cultured overnight, then processed for mRNA and protein analysis by RT-PCR and immunofluorescence assay.

Spleen T cell Preparation and Culture

Single cell suspensions of splenic leukocytes were obtained by gently pressing freshly explanted spleens through wire mesh screens followed by a single wash in media. Erythrocytes were lysed in a 0.84% ammonium chloride treatment. The remaining leukocytes were washed twice with media. Purified T lymphocytes were obtained by passing suspensions of spleen cells through a nylon wool fiber column. T cell purification columns were pre-prepared by saturating and washing 0.6 g nylon wool fiber with 50 ml of media in a 12 cc syringe followed by incubation with media for 1 hour at 37^0C in a 5% CO_2 atmosphere, and then washed with 20 ml media again. Approximately 3 ml (40~150x10^6 cells) cells were added to the column, allowed to penetrate into column, and incubated at 37^0C in a 5% CO_2 atmosphere for 45 min. Cells were then collected in 10 ml of media. For MP and T cell co-culture, 5x10^5 T cells were added to 1x10^5 MPs cultured in 96 well culture plates.

Reverse Transcriptase/Polymerase Chain Reaction (RT-PCR) Analysis of PGS-2 and other Cytokine mRNA

Poly (A^+) mRNA from $1x10^5$ MPs, $1x10^4$ DCs, $5x10^5$ T cells, or same number of cells from T/MP, T/DC, T/MP/DC co-culture were obtained by using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). Isolated mRNA was reverse transcribed at 42^0C for 1 hr in 20ul reaction mixtures containing 400 units of Moloney Murine Leukemia Virus reverse transcriptase. The resultant cDNAs were subjected to PCR (35 cycles in a Gene Machine II, programmable thermal cycler, USA/Scientific Plastics, Ocala, FL). The oligonucleotide PCR primers specific for PGS-2 were derived from exon 9 (5'-primer, 5'-CAAGCAGTGGCAAAGGCCTCCA-3') and exon 10 (3'-primer, 5'-GGAACTTGCATTGATGGTGGCT-3'). G3PDH primers were used as an assay control. Each PCR cycle consisted of denaturation for 45 seconds at 94^0C, annealing for 45 seconds at 60^0C and extension at 72^0C for 2 minutes, followed by 7 minutes at 72^0C for final extension. The 50 ul of reaction mixture contained 50 pM of each primer, 1.25 U of AmpliTaq DNA polymerase (Boehringer Mannheim, Germany) and 2 ul of cDNA. Fifteen microliters of each 50 ul PCR reaction were resolved by electrophoresis through a 1.4% agarose gel that was subsequently stained with ethidium bromide. PCR results were confirmed by Southern blotting using a digoxigenin labeled internal probe.

Oligonucleotides derived from PGS-2 exon 10 (5'-GTGCTCCAAGCTCTACCA-3') were labeled with digoxigenin-labeled uridine-triphosphate (Dig-UTP) using the enzyme terminal transferase as per instructions from Boehringer Mannheim. Probes were hybridized to positively charged nylon membranes to which PCR gels had been blotted. Detection of hybridized probes revolves around the use of an anti-DIG alkaline

phosphatase conjugate (DIG-AP). After DIG-AP is reacted with any hybridized probes, a subsequent AP-catalyzed color reaction with 5-bromo-4-chloro-3-indoyl phosphate (x-phosphate) and nitroblue tetrazolium salt (NBT) as substrate to produces an insoluble blue precipitate. The presence of blue bands is indicative of the presence of probe-PCR product hybrid molecules.

The primer and probe sequences of other cytokines were designed from published sequences (Anderson, 1991). All primers and probes were synthesized in the University of Florida ICBR DNA Synthesis Laboratory (Gainesville, FL).

Immunofluorescence Analysis of PGS-2 Protein

MPs ($1x10^5$) were purified in multi-chamber culture slides by the method described above. After 16 hr of culture, the MPs were fixed in 2% paraformaldehyde/PBS for 30 min. The fixed cultures were rinsed with PBS and further washed in PBS-GT (0.1M glycine, 0.05% Triton X-100). MPs were incubated in normal goat serum (1:20 dilution) for 30 minutes, then overnight at 4^0C with anti-PGS-2 antibody (1:250 diluted in PBS- Tween (0.2%)). After three 10-min washes with PBS-T, the cultures were washed three times for 20 min each, dried, mounted in buffered glycerol containing 1 mg/ml paraphenylenediamine, and analyzed with a Zeiss Photomicroscope III. Exposures were for 8 sec in all cases, using Kodak Tri-X Pan 400 film. Magnifications were 100x, in all cases (Reddy et al., 1994).

SDS-Polyacrylamide Gel Electrophoresis and Western Analysis

Fresh peritoneal MPs were obtained by method described above. Cells were washed three times with PBS, and harvested in SDS gel loading buffer (50 mM Tris-

HCL (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol). Samples were incubated for 10 min at 100°C, sonicated, and protein concentration was determined. 75 μg of the protein extract was analyzed on SDS-polyacrylamide electrophoresis (5% stacking and 8% resolving gel), using a tris-glycine buffer (pH 8.3). The proteins were electroblotted using BioRad's Mini Protein II according to the manufacturer's procedures. Filters were then incubated in PBS, 0.2% Tween 20, 10% non-fat milk for 60 min, washed with PBS buffer, and incubated with primary and secondary antibodies as suggested in the manufacturer's protocols. Dilution's of 1:6000 and 1:8000 for primary and secondary antibodies, respectively were sufficient for immunodetection using the enhanced chemiluminescence reagents. The filters were exposed to Kodak XAR-5 film at room temperature (Reddy et al., 1994).

PGE-2 Analysis

Peritoneal MPs ($1x10^5$) or Peritoneal MPs ($1x10^5$) plus T cells ($5x10^5$) were cultured for 24 hours or time specified with or without NS-398 and in the presence or absence of LPS (10 ug/ml) in RPMI 1640 and 2% endotoxin free fetal bovine sera culture media. Supernatants were harvested for PGE-2 analysis by using a PGE-2 specific EIA kit (Cayman Chemical). Results are expressed as pg/ml PGE-2/10^5 MPs.

Spleen Cell Preparations and Flow Cytometric Analysis

Single cell suspensions of splenic leukocytes from SEB immunized NOD, BALB/c and C57BL/6 mice were obtained as described above. The collected leukocytes were washed twice with media, then, $1x10^6$ cells were resuspended into FACS buffer (1xPBS with 1% bovine serum album and 0.1 % sodium azide) and labeled with either

FITC conjugated TCR Vβ6 or 8 specific antibodies (Pharmingen, San Diego, CA). After 45' min at 4°C, the cells were, and then incubated with PE conjugated anti-CD4 antibody (Pharmingen, San Diego, CA) at 4°C for another 45 min. Then, cells were washed in FACS buffer and used for two color FACS analysis. Single color FACS using Vβ6 TCR, Vβ8 TCR, CD4 antibodies were also analyzed. FITC and PE labeled isotype matched antibodies were used as controls.

For FACS, viable cells (10,000/analysis) were gated using a combination of forward and side scatters and analyzed on a FACScan Flow Cytometer (Becton-Dickinson, Mountain View, CA) using logarithmic scales. TCR Vβ8+/CD4+ and Vβ6+/CD4+ double positive cells were determined by two color fluorescent analysis. Data were analyzed using PC Lysis software. Results are expressed as the percentage of Vβ 8+/CD4+ or Vβ 6+/CD4+ cells divided by total CD4+ cells.

Syngenic Mixed Lymphocyte Reaction (SMLR) Assay

Splenic cells as antigen presenting cells and purified splenic T cells were isolated as described above. Single cell suspensions of dendritic cells were prepared by passage axillary and inguinal lymph node through Nitex 110 mesh, then purified by metrizamide gradient. MPs were isolated by overnight adherence. B cells were isolated by using mouse anti-IgG coupled magnetic beads.

SMLR responses were determined in triplicate in flat-bottomed 96-well, in a final volume of 0.2 ml/well, containing 5×10^5 nylon wool-purified T cell/well cultured in medium alone or with DCs (1×10^4), MPs (1×10^5), or splenic cells (APCs, 1×10^5) with or without MPs or B cells. Splenic APCs (with or without MPs and B cells) were

irradiated at 1500 Rads before used for co-culture. Microtiter plates were incubated for 5 days at 37^{O}C in a 5% CO_2/95% air humidified atmosphere. For the final 18 h of culture, each well was incubated in the presence of 0.5 μCi/well of [^{3}H] thymidine (New England Nuclear, Boston, MA) and cultures were harvested onto glass fiber disks by a cell harvester (Cambridge Technology, Cambridge, MA). The disks were then counted in 3 ml of Aquasol-2 (New England Nuclear). Data are presented as the mean cpm of triplicate co-cultures of T cells and DCs, MPs, or APCs divided the mean cpm of triplicate T cell cultured alone.

PGS-2 Promoter Sequence Analysis

Genomic DNA was extracted from $2x10^{6}$ rest peritoneal macrophages of NOD and BALB/c mice using Qiagen genomic DNA isolation kit (Qiagen Inc., Chatsworth, CA). Two microliters genomic DNA from 20 ul total was subjected to PCR (35 cycles in a Gene Machine II, programmable thermal cycler, USA/Scientific Plastics, Ocala, FL). Two pairs of overlapping oligonucleotide PCR primers specific for the PGS-2 promoter were used. The first pair of primers: 5'-primer, 5'-GGCCAACACAAACACAGTAGG-3', 3'-primer, 5'-TCCCTCCCGGGATCTAAG-3' covers -1 to -600 bp of the PGS-2 promoter sequence; and the second pair of primers: 5'-primer, 5'-AGTGGGGAGAGGTGAGGG-3', 3'-primer, 5'-TCTTTGCCAATACACAGCCA-3' covers -500 to -1000 bp PGS-2 promoter sequence. Together, these primers covered the 5' promoter region from -996 nucleotide to the transcriptional start site). Each PCR cycle consisted of denaturation for 45 seconds at 94^{0}C, annealing for 45 seconds at 60^{0}C and extension at 72^{0}C for 2 minutes, followed by 7 minutes at 72^{0}C after all cycles were

finished. The 50ul of reaction mixture contained 50 pM of each primer, 1.25 U of AmpliTaq DNA polymerase (Boehringer Mannheim, Germany) and 2ul of genomic DNA. PCR products were purified by 1.4% agarose gel electrophoresis and sequenced at the University of Florida sequence core laboratory (Gainesville, FL) by using the cycle sequencing procedure and an ABU 373 automated sequencer.

Statistical Analyses

All measures of variance are given as SEM of the mean. Tests of significance for difference in SMLR response and PGE-2 production in different group were performed with the one-way analysis of variance (ANOVA). Results in which $p < 0.05$ were considered significant. Test of significant for difference in incidence of diabetes between NOD mice group treated with indomethacin/aminoguanidine or indomethacin alone and untreated NOD mice group were performed with logrank test chi-square. $p < 0.05$ were considered significant. p values are one sided.

CHAPTER 3
RESULTS

Effects of Macrophages on Dendritic Cell-Mediated T cell Activation in NOD Mice

The syngeneic mixed lymphocyte reaction (SMLR) has been characterized as an *in vitro* activation of CD4+ T lymphocytes recognizing class II molecules on the surface of syngeneic antigen presenting cells (APC) (Glimcher et al, 1981). The murine SMLR, provides a model system for studying immunoregulatory mechanisms *in vitro*. Previous studies in our lab and in others have shown that there is a depressed syngeneic mixed lymphocyte reaction in NOD mice (Serreze et al, 1988) and a defect in the APC stimulation of T cell proliferation has also been suggested (Yokona et al, 1989). In those experiments, splenic cells were used as APCs to stimulate syngeneic T cells. Spleen cells, however, contain a mixture of APC sub-populations including dendritic cells (DCs), macrophages (MPs), and B cells. To investigate which sub-population of APCs specifically contributed to the decreased ability of APCs to activate T cells in NOD mice, we purified APC populations and stimulated syngeneic T cells. We established that 5 days of culture is the peak response time for the SMLR stimulated by DC, MPs, and B cells. As shown in figure 3, we first demonstrated that the suppression of SMLR is not due to DC dysfunction as we found that lymph node (LN) DCs from NOD mice were paradoxically better stimulators of syngenic T cell response than DCs from control mice. Also NOD MPs and purified spleen B cells are similar to control strains in their capacity

to stimulate T cells. However, the mixed spleen APC populations are deficient, suggesting the interaction of sub-populations of APCs may disturb the ability of other APCs to activate T cells. We therefore subtracted B cells by anti-IgG magnetic bead separation , and MPs by overnight adhesion. We found that the removal of the MP population from splenic cells markedly increased the stimulatory capacity of splenic cells to T cells in NOD mice, but not in control strain BALB/c mice (figure 4). Furthermore, when MPs were added back to DCs and T cells, marked suppression of the SMLR, once again occurred (figure 5). In transwell experiments, we established that the effect of MPs was mediated through soluble factors and have gone on to demonstrate that NO and PG are the chief mediators of T cell suppression as it is reversed completely by the NO and PG inhibitors NMMA and indomethacin (figure 5). These results demonstrated that MPs suppress syngeneic T cell activation when in the environment of a strong immune response such as that provided by DCs.

To further analyze the T cell activation and APC contribution in SMLR, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, TGF-β, TNF-α, IFN-γ, iNOS, and PGS-2 mRNA expression were analyzed in MPs ($1x10^5$ cells), DC ($1x10^4$ cells), T cells ($5x10^5$ cells), T ($5x10^5$ cells)/MPs ($1x10^5$ cells), T ($5x10^5$ cells)/DCs ($1x10^4$ cells), and T ($5x10^5$ cells)/DCs ($1x10^4$ cells)/MPs ($1x10^5$ cells) following 24 hr cell culture by RT-PCR assay. For T/DC/MP co-culture, mRNA expression was assessed in the presence or absence of indomethacin (I) and N-monomethyl-L-arginine (NMMA).

After examining different mRNA expression in the single cell population culture or combined cell population co-culture, it was found that cells from the BALB/c T cells

($5x10^5$ cells)/DC ($1x10^4$) cells)/MPs ($1x10^5$ cells) co-culture expressed IL-2, IL-4, IL-12, TNF-α, IFNγ, TGF-β, but not PGS-2 and iNOS mRNA (Table 1). In contrast, in the same culture system, cells from NOD mice expressed IL-1β, PGS-2 and iNOS (Table 2). The absence of IL-2/IL-4 expression in NOD is consistent with previous studies (Serreze et al., 1993), and high levels of PGS-2, iNOS, and IL-1β expression in NOD suggests the MPs are activated in SMLR. Studies using NOD DC/MP/T co-cultures also showed that addition of PG and NO inhibitors enhanced IL-2 expression and suggests that PG/NO may suppress T cell activation through down-regulation of IL-2 or IL-2R expression.

The key finding in these experiments, however, was the differential expression of PGS-2 in MPs of NOD and control strain mice. After examining purified resting peritoneal MPs, I found that PGS-2 is consititutively expressed in MPs of female NOD mice but not in BALB/c MPs. The constitutive expression of PGS-2 in NOD MPs strongly suggested that the NOD MPs are "activated" as this enzyme is reported to be expressed only in activated Mps.

Spontaneous PGS-2 Expression in Macrophage of NOD and NODscid/scid Female Mice

In above mentioned experiments, I found that PGS-2 mRNA is consititutively expressed in NOD MPs but not in MPs of control mice. However, PGS-2 mRNA expression can be induced successfully in control MPs by overnight LPS stimulation (figure 6). Although endotoxin contamination could upregulate PGS-2 expression in NOD mouse, this is unlikely as BALB/c MPs cultured in the same media did not express PGS-2. Additional proof that NOD PGS-2 expression is not due to endotoxin

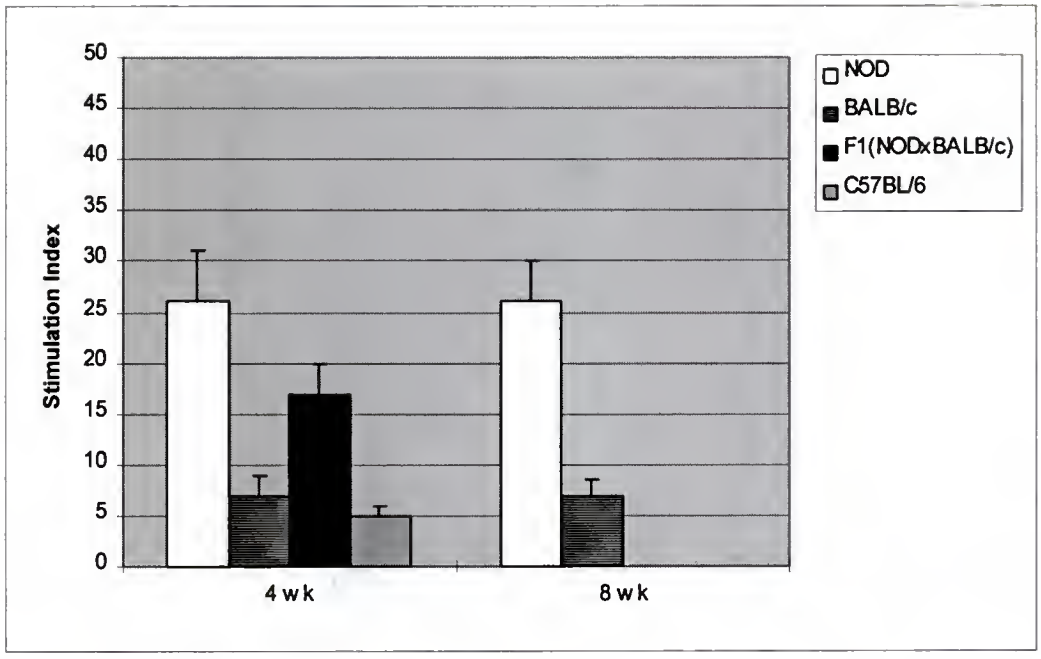


Figure 3. Lymph node dendritic cells (DCs) stimulation of syngeneic T cell response. LN DCs ($1x10^4$) from 4 wk and 8 wk old female NOD and control mice were purified by metrizamide gradient and used to stimulate nylon wool purified splenic T cells ($5x10^5$). DCs/T cells were co-cultured for 5 days and proliferation was assessed by [^{3}H]thymidine uptake in the last 18 hr of culture. Results are expressed as mean value of stimulation index ± SEM (n=10 experiments, $p< 0.05$) (Stimulation index is the mean value of the cpm from triplicate DC/T cultures divided by mean value of cpm from T cells alone).

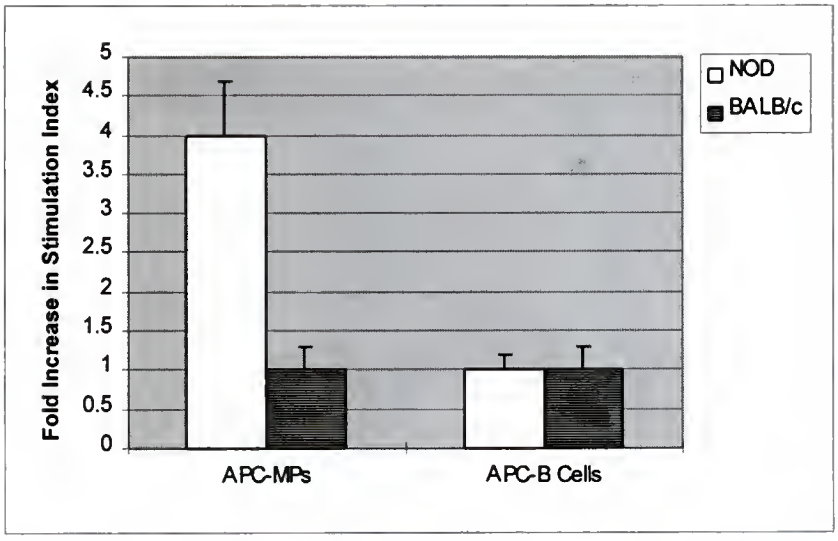


Figure 4. Syngeneic T cell response stimulated by antigen presenting cell (APC) without macrophage (MP) or B cell. The subtraction of MPs from the spleen by overnight adherence markedly enhances the stimulation of T cells from the 8 week old NOD female mice, but not from spleen cells of age matched control BALB/c female mice. B cell subtraction by mouse anti-IgG coupled magnetic beads did not affect T cell response in either strain. APCs ($1x10^5$ cells) without MPs and B cells were irradiated at 1500 Rads and then cultured with $5x10^5$ nylon wool purified T cells. APCs/T cells were co-cultured for 5 days and proliferation was assessed by [^{3}H]thymidine uptake in the last 18 hr of culture. Results are expressed as mean value of the fold increase of stimulation index ± SEM (n=10 experiments, $p< 0.05$) (fold increase of stimulation index is expressed as stimulation index of splenic APCs without MPs or B cells divided by stimulation index of intact splenic APCs

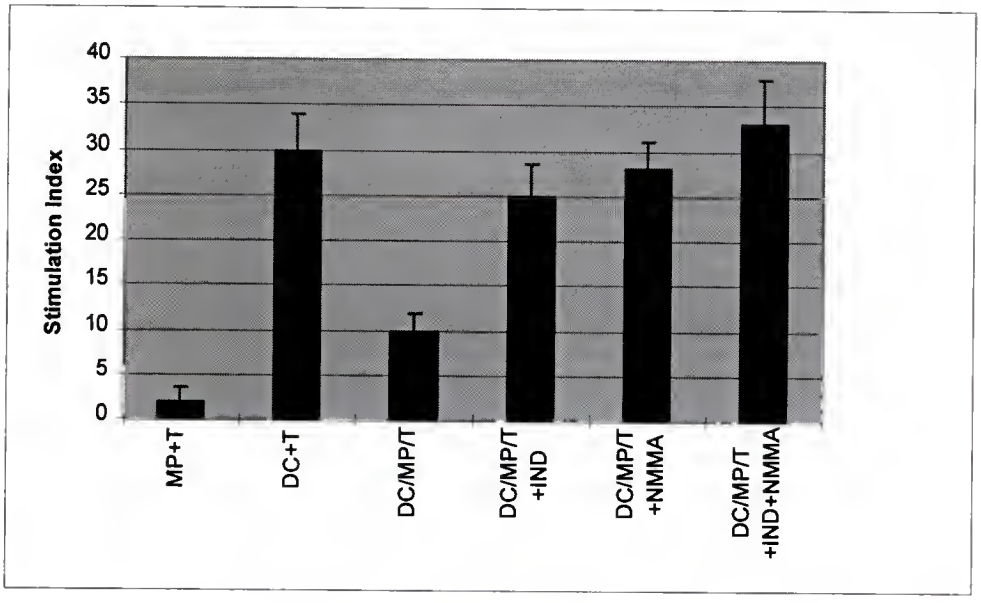


Figure 5. Effects of iNOS and cyclooxygenase inhibitors on the MP suppression of SMLR in 8 week old female NOD mice. Metrizamide gradient purified LN DCs ($1x10^4$) and overnight adherence purified MPs ($1x10^5$) alone or combinations were used to stimulate splenic T ($5x10^5$) cell activation in the presence or absence of 1 mM indomethacin (I) and/or N-monomethyl-L-arginine (NMMA). Cells were cultured for 5 days and proliferation was assessed by [^{3}H]thymidine uptake in the last 18 h of culture. Results are expressed as mean value of stimulation index ± SEM (n=10 experiments, $p< 0.05$).

Table 1. Summary data of cytokine mRNA expression in cell cultures of 6-8 week old female BALB/c mice. Lymph node dendritic cells (DC, $1x10^4$), peritoneal macrophages (MP, $1x10^5$) and splenic T cells (T, $5x10^5$) by themselves or in combination were cultured for 24 hours. DC/MP/T were cultured in the presence or absence of indomethacin (I) (13 ug/ml) and N-monomethyl-L-arginine (NMMA) (250 ug/ml). After 24 hr culture, cells were harvested and mRNA were extracted by using mRNA isolation kit and amplified by RT-PCR followed by Southern blotting using a digoxigenin labeled cytokine specific internal probe. (N=2 set of experiments, "++" : PCR product visible on the gel (further verified by blot), "+" : PCR product visible on the blot, "-" : PCR product undetectable on the blot)

	T	DC	MP	T/DC	T/MP	DC/MP/T	DC/MP/T +I	DC/MP/T +NMMA	DC/MP/T +I+NMMA
β-actin	++	++	++	++	++	++	++	++	++
IL-2	—	—	—	+	+	+	+	+	+
IL-4	—	—	—	—	—	+	+	+	+
IL-12	—	—	—	+	—	+	+	+	+
IFN-γ	+	—	—	+	++	++	+	+	+
TNF-α	—	—	—	—	+	+	+	+	+
TGF-β_3	+	—	—	+	+	+	+	+	+
IL-1β	—	—	+	—	+	+	+	+	+
iNOS	—	—	—	—	—	—	—	—	—
PGS-2	—	—	—	—	—	—	—	—	—

Table 2. Summary data of cytokine mRNA expression in cell cultures of 6-8 week old female NOD mice. Lymph node dendritic cells (DC, $1x10^4$), peritoneal macrophages (MP, $1x10^5$) and splenic T cells (T, $5x10^5$) by themselves or in combination were cultured for 24 hours. DC/MP/T were cultured in the presence or absence of indomethacin (I) (13 ug/ml) and N-monomethyl-L-arginine (NMMA) (250 ug/ml). After 24 hr culture, cells were harvested and mRNA were extracted by using mRNA isolation kit and amplified by RT-PCR followed by Southern blotting using a digoxigenin labeled cytokine specific internal probe. (N=3 set of experiments, "++" : PCR product visible on the gel (further verified by blot), "+" : PCR product visible on the blot, "-" : PCR product undetectable on the blot)

	T	DC	MP	T/DC	T/MP	DC/MP/T	DC/MP/T +I	DC/MP/T +NMMA	DC/MP/T +I+NMMA
β-actin	++	++	++	++	++	++	++	++	++
IL-2	—	—	—	+	—	—	—	—	—
IL-4	—	—	—	—	—	—	—	—	—
IL-12	—	—	—	+	—	+	+	+	+
IFN-γ	+	—	—	+	—	+	+	+	+
TNF-α	—	—	+	+	+	+	+	+	—
TGF-β_3	—	—	—	—	—	—	—	—	—
IL-1β	—	—	—	+	+	++	++	++	++
iNOS	—	—	—	—	—	+	+	+	+
PGS-2	—	—	++	—	++	++	++	++	++

contamination comes from experiment where polymixin B, a specific endotoxin inhibitor, was added to NOD MPs cell cultures and it did not block PGS-2 expression. In order to established that the gene is truly expressed constitutively, I evaluated the kinetics of PGS-2 mRNA expression in MPs of NOD and control strains. I harvested mRNA from cells immediately after peritoneal lavage, as well as after 1, 2, 6, 12, 24, and 48 hr of cell culture. I found that PGS-2 is expressed at all time points in the MPs of NOD mice, but is not expressed at any time points in the MPs of control strains (BALB/c and C57BL/6 mice).

PGS-2 protein expression was also examined in the resident peritoneal MPs of 6-8 weeks old female NOD, BALB/c and C57BL/6 mice. Since previous reports suggested that PGE-2 production from MPs can be upregulated by exposure to female sex steroid hormones (El Attar et al., 1982; Smith et al., 1986; Yagel et al., 1987), I examined PGS-2 protein expression at various points during the estrus cycle as determined by vaginal smear. I found that PGS-2 protein expression in MPs of female NOD mice is related to the phase of the estrus cycle and can be detected only in the MPs of estrus phase mice. In contrast, I did not detect any PGS-2 protein expression at any point of the estrus cycle of control mouse strains (BALB/c, C57BL/6) (figure 7). The kinetics of PGS-2 protein expression in MPs of estrus phase NOD female mice demonstrated that the PGS-2 protein is constitutively expressed at all time points; i.e., immediately after peritoneal lavage, as well as after 2, 6, 12, 24, and 48 hours of cell culture. In contrast, PGS-2 protein expression was not detected at any time points in the MPs of control mice.

The spontaneous expression of PGS-2 in NOD MPs could caused by either a primary defect in the regulation of PGS-2 gene itself or secondary to MP activation by the autoimmune environment. To investigate whether the autoimmune milieu in NOD mice contributes to PGS-2 expression, we examined MP PGS-2 mRNA and protein expression in female NODscid/scid mice. The NOD scid/scid mouse, is a congenic strain genetically identical to NOD but lacking functional T and B cells due to a homologous mutation at the severe combined immunodeficiency (SCID) locus. NODscid/scid mice do not develop autoantibodies, insulitis or diabetes. We examined PGS-2 mRNA (figure 8) and protein (figure 9) expression in unstimulated peritoneal macrophages of 6-8 wk old female NODscid/scid mice and found that PGS-2 mRNA and protein expression were readily detectable, as in NOD MPs. The spontaneous expression of PGS-2 in the NODscid/scid mice suggests that functional lymphocytes and active autoimmunity are not required for MP expression of PGS-2 in NOD mice. This data supports the idea that the PGS-2 enzyme is spontaneously expressed in estrus female NOD MPs and suggests that the NOD PGS-2 gene regulation differs substantially from control strains.

Aberrant PG Metabolism in NOD, NODscid/scid, and Congenic Mice

PGS-2 is the rate limiting enzyme in PG metabolism and is responsible for the production of large quantities of PGs. Since PGS-2 is consititutively expressed in NOD mice, I hypothesized that there is enhanced PG production by NOD MPs. Examination of PGE-2 production from resting peritoneal MPs of NOD and control mice in cell culture revealed that PGE-2 production, as measured in the supernatant of $1x10^5$ unstimulated

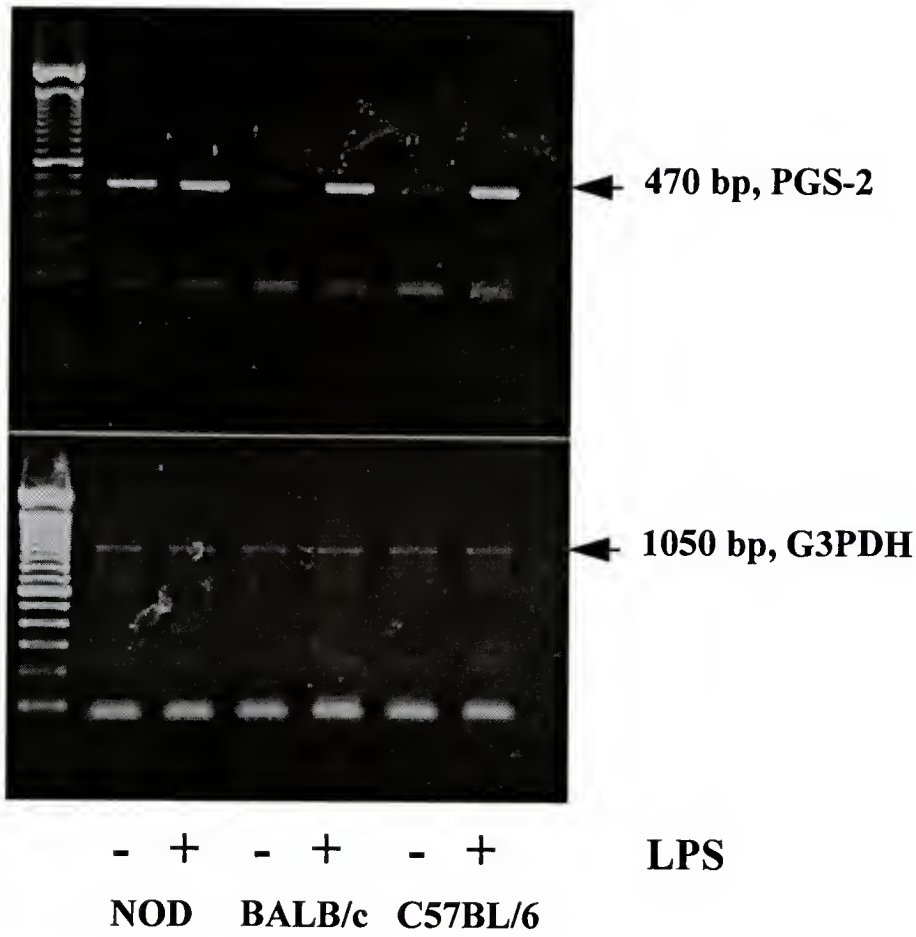


Figure 6. PGS-2 mRNA expression in estrus NOD, BALB/c and C57BL/6 mice. mRNA expression was assessed in 1 x 10^5 resident peritoneal MPs from 8 weeks old female mice by RT-PCR after overnight culture with and without LPS stimulation. Unstimulated 8 weeks old BALB/c and C57BL/6 mice do not spontaneously express PGS-2 mRNA, but do so when stimulated *in vitro* by LPS (10 ug/ml). PGS-2 and G3PDH PCR product were detected on the 1.4% agarose gel (verified by blot). This is a representative PCR result. (NOD, n=16 mice examined; BALB/c, n=16; C57BL/6, n=8). G3PDH mRNA was used as an internal positive control and was equally expressed in all samples analyzed.

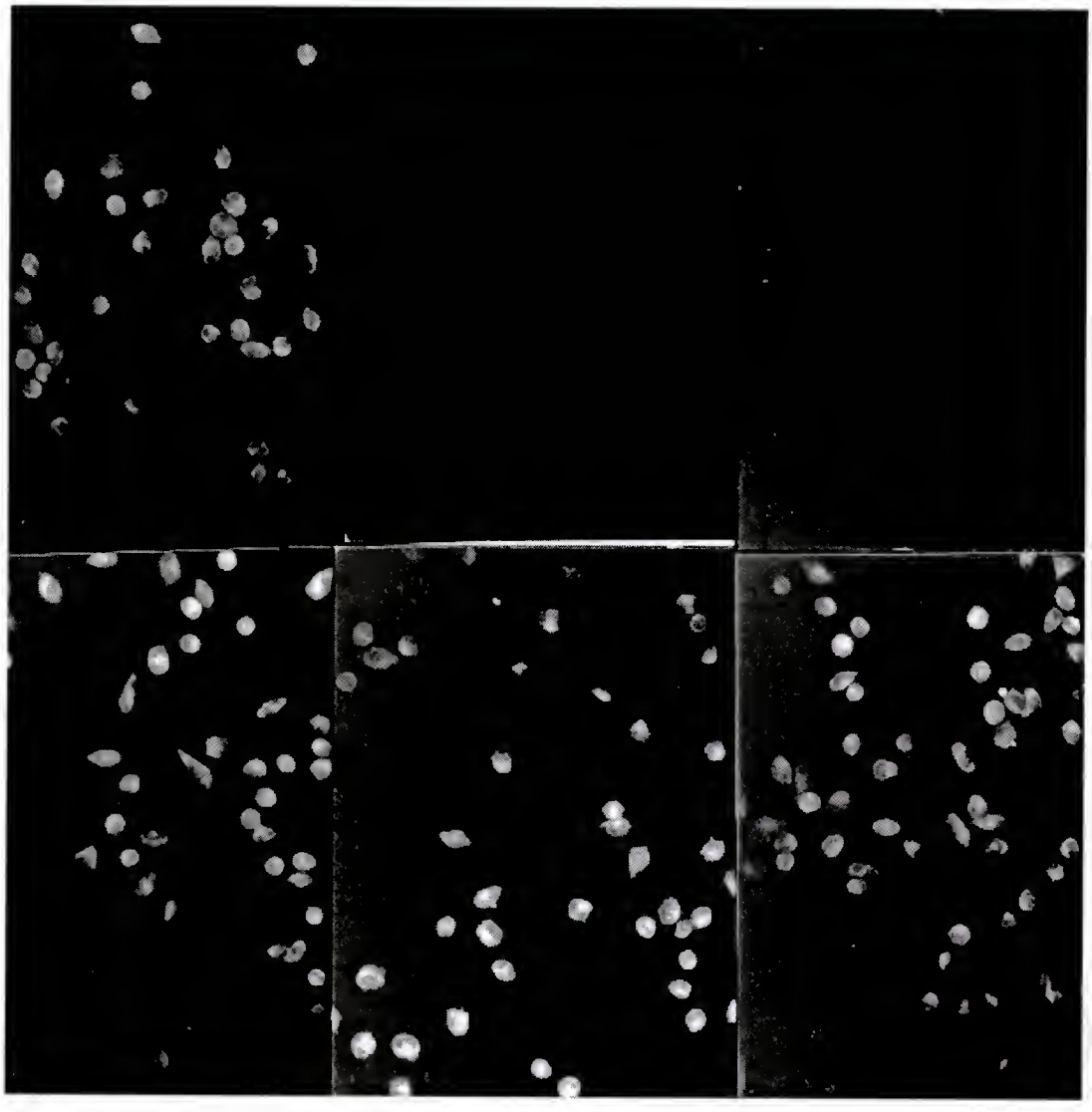


Figure 7. Expression of PGS-2 protein in the resident peritoneal MP of 8 week old estrus NOD, BALB/c and C57BL/6 mice. PGS-2 was detected by indirect immunofluorescence using a specific PGS-2 specific anti-sera (Herschman's laboratory, UCLA; Reddy et al., 1994) after MPs were cultured for 16 hours with or without LPS stimulation (10 ug/ml). NOD MPs spontaneously expressed PGS-2 protein without LPS stimulation. In contrast, BALB/c and C57BL/6 MPs did not express PGS-2 protein, but it was readily induced by LPS stimulation. There is no apparent increase in PGS-2 expression in NOD MPs after LPS stimulation.

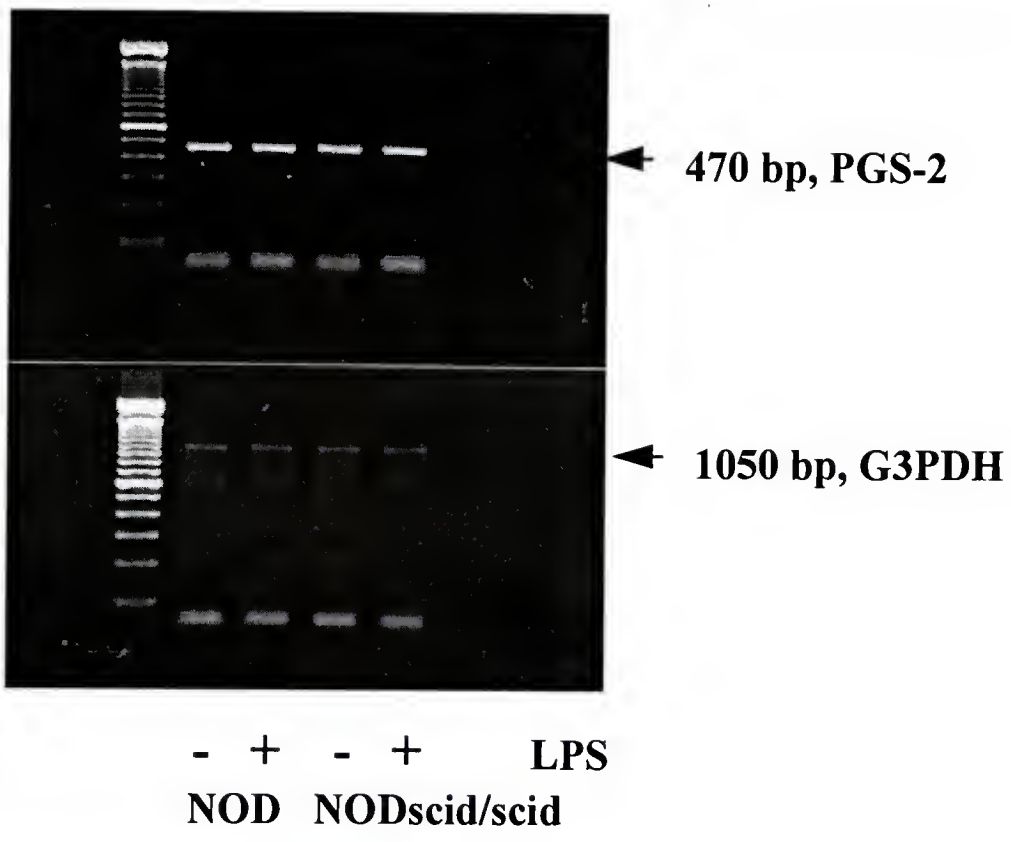


Figure 8. PGS-2 mRNA expression in estrus NOD, NODscid/scid mice. mRNA expression was assessed in 1 x 10^5 resident peritoneal MPs from 8 weeks old female mice by RT-PCR after overnight culture with and without LPS (10 ug/ml) stimulation. The NODscid/scid mouse, which does not contain functional T and B lymphocyte, and does not develop active autoimmune disease, expresses PGS-2 mRNA in a manner identical to NOD. LPS stimulation does not markedly upregulate mRNA expression in NOD and NOD scid/scid mice. PGS-2 and G3PDH PCR product were detected on the 1.4% agarose gel (verified by blot). This is a representative PCR result. (NOD, n=16 mice examined; NODscid/scid, n=10).

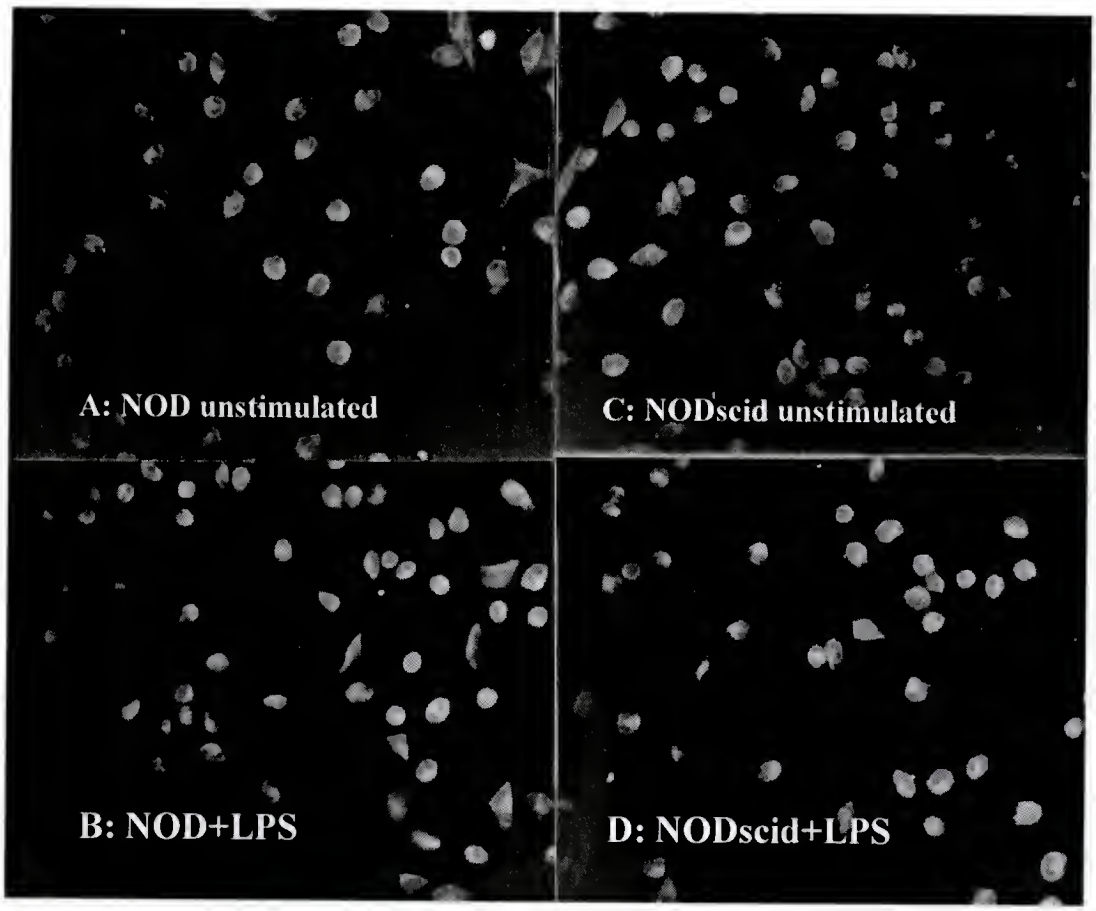


Figure 9. Expression of PGS-2 protein in the resident peritoneal MPs of 8 week old estrus NOD and NODscid/scid mice. PGS-2 was detected by indirect immunofluorescence using a specific PGS-2 anti-sera (Reddy et al., 1994) after MPs were cultured for 16 hours with or without LPS stimulation (10 ug/ml). NOD and NOD scid/scid MPs spontaneously express PGS-2 protein without LPS (10 ug/ml) stimulation and there is no demonstrable change of PGS-2 expression in NOD or NODscid/scid MPs after LPS stimulation.

resident peritoneal NOD MPs cultured overnight, was consistently higher than MPs from control mice (figure 10).

To evaluate the influence of the autoimmune environment on PGE-2 production in NOD mice, I also examined PGE-2 production from MPs of NODscid/scid mice (PGS-2 positive). I found that PGE-2 production from NODscid/scid MPs was similar to NOD mice (figure 10). I also examined PGE-2 production by MPs of congenic strain, NOD.B10C1 mice. This mouse has an NOD background with a large interval of chromosome 1 containing the PGS-2 gene from C57BL/10snj mice (detailed information about this mouse and its PGS-2 expression will be discussed later in this chapter). This mouse is negative for PGS-2 mRNA and protein expression, and its PGE-2 production is lower than NOD (figure 10). We further demonstrated that enhanced PGE-2 production by NOD MPs is mediated by the PGS-2 enzyme, as the PGS-2 specific inhibitor, NS-398, completely blocks its production (figure 10). These data demonstrated that enhanced PG production in NOD MPs is mediated through PGS-2 and not due to the autoimmune environment. Although there is no statistically significant difference ($p= 0.08$) in PGE-2 production between MPs of NOD and control strains mice, the consistently higher PGE-2 production from NOD MPs may contribute to the biologically functional differences between NOD and control strains of mice. In control MPs, NS-398 reduced PGE-2 production to a small degree. This is most likely due to the transient expression of PGS-2 that occurs following the adherence of control MPs, as previously published studies suggest (DeWitt et al., 1993).

Since PGE-2 production potently affects the activation of T cells in DC/T/MP co-culture system as previous discussed, I hypothesized that a T cell or perhaps a DC factor may contribute to the enhanced production of PGE-2 by MPs. Analysis of PGE-2 production from the co-cultures of MP/T or DC/MP/T in NOD and control strains mice was under taken to test this theory.

I found that T cells enhanced PGE-2 production by MPs in all mice strains tested, but PGE-2 production from NOD MP/T cells co-culture was consistently higher than MP/T cell cultures from control strains. Furthermore, enhanced PGE-2 production was mediated by PGS-2, since NS-398 totally blocked PGE-2 production (figure 11). The production of PGE-2 by NOD.B10C1 MPs which are negative for PGS-2 expression was also examined in co-culture. NOD.B10C1 MPs were cultured in the presence of both NOD and NOD.B10C1 T cells and found to have PGE-2 production similar to controls. These data suggest that NOD T cells by themselves are not capable of stimulating the higher PGE-2 production seen in NOD, and that PGS-2 phenotype in NOD is essential for enhanced PGs production. PGE-2 production in the DC/ MP/T co-culture were found to be even higher in all mice strains examined, again, NOD DC/MP/T co-cultures are higher in PGE-2 production than control mice strains and the difference is significant ($P < 0.04$) (figure 12).

These data suggest that signals from T cells, which may be enhanced by the presence of DC, contribute to a general upregulation of PGE-2 production. The mechanism involved is still largely unknown but Wang et al (Wang et al., 1996) found that leukoregulin, a 50-kD cytokine product of mitogen-activated T lymphocytes, can

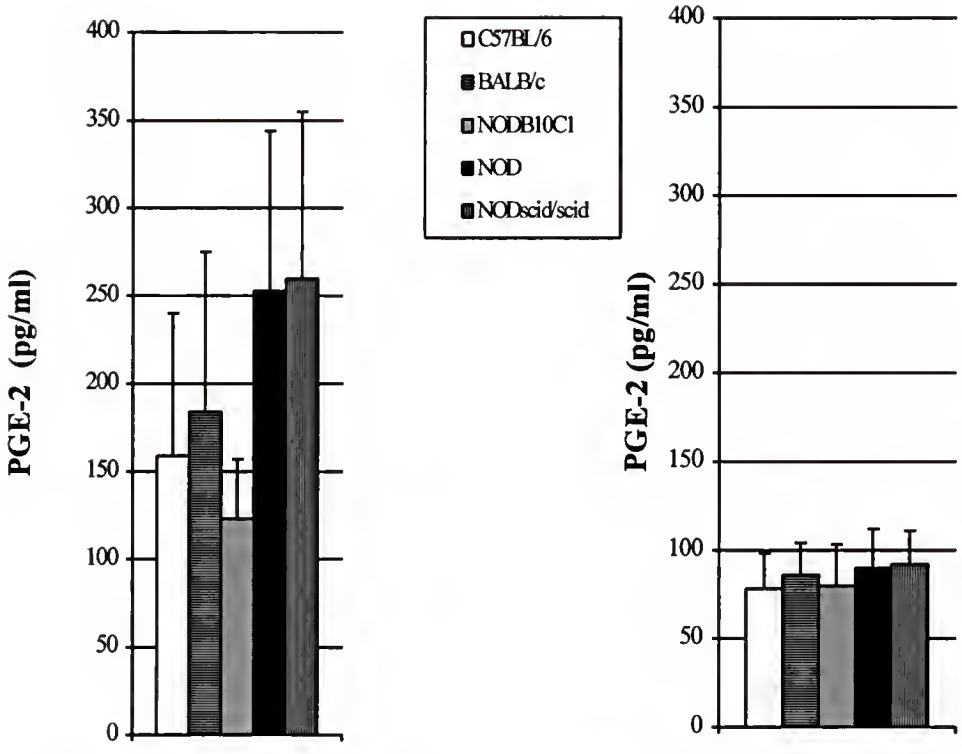
dramatically increase PGE-2 synthesis in cultured human orbital fibroblasts. This up-regulation is mediated through a substantial increase in steady-state PGS-2 mRNA and protein levels. This in turn results markedly increased generation of cAMP plus an alteration in orbital fibroblast morphology. In the murine macrophage, leukoregulin could play a similar role in the transient activation of PGS-2 and upregulation of PGE-2 production in both the NOD and control strains.

Factor(s) which Influence PGS-2 Gene Expression and the Mechanism of Spontaneous PGS-2 Expression in NOD Mice

NOD PGS-2 Promoter Sequence Analysis

The PGS-2 regulatory region has several consensus sequences for transcriptional activation including two potential NF-IL6 response elements, a Sp1 consensus sequence, two cyclic AMP (cAMP) response elements (ATF/CRE), and an E box. Defects in any regulatory element may potentially affect PGS-2 gene expression.

To investigate the contribution of cis-regulatory elements on PGS-2 expression in the NOD mice, 5' promoter region was sequenced from -966 nucleotides to the transcriptional start site. Genomic DNA from MPs of female NOD mice was extracted from MPs and the PGS-2 promoter sequence was amplified the by PCR. PCR amplified products were sequenced in the University of Florida sequence core laboratory (Gainesville, FL). Sequence analysis showed that there were no mutations in the NOD PGS-2 promoter compared with PGS-2 promoter from C57BL/6 or the published sequence of 3T3 fibroblast cells (Fletcher et al., 1992). Christy Myrick in Dr. Wakeland's laboratory (University of Florida, Gainesville, FL) further confirmed our results by cloning the NOD and C57BL/6 PGS-2 promoter gene into a TA vector system



A: MPs **B: MPs+NS398**

Figure 10. PGE-2 production by murine macrophages. PGE-2 production from cell culture (MEM media without phenol red and with 2% fetal bovine serum) of estrus phase resident peritoneal MPs ($1x10^5$) of 8 week old female NOD, NODscid/scid, congenic, and control mice was detected by ELISA (ELISA kit, Cayman Chemicals) after MPs were cultured 16 hours with and without a PGS-2 specific inhibitor, NS-395 (5 uM). Values are given as the mean ± SEM. NOD, n=6 experiments; BALB/c, n=6; C57BL/6, n=5; NODscid/scid, n=4; NOD.B10C1, n=4. One-way ANOVA was used to compare the means of PGE-2 production in different MPs, p= 0.0795.

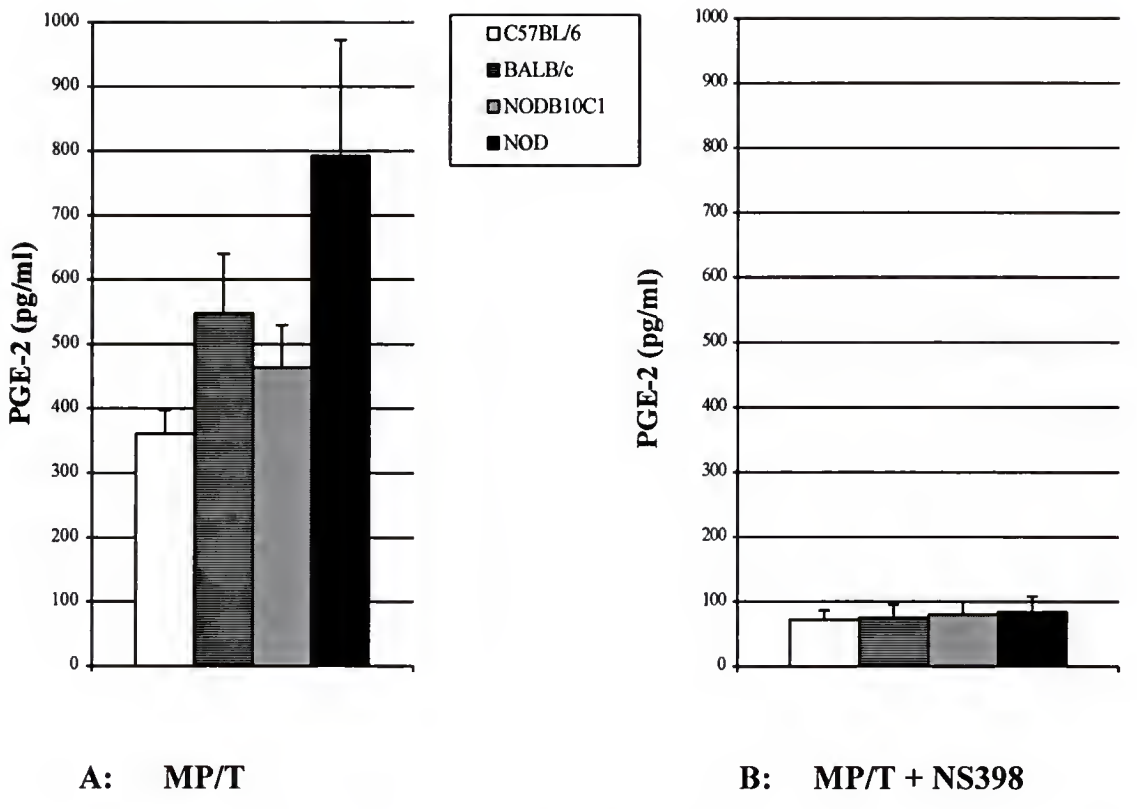


Figure 11. PGE-2 production by murine macrophage and T cell co-culture. PGE-2 production from MP ($1x10^5$) /T cell ($5x10^5$) co-culture (MEM media without phenol red and with 2% fetal bovine serum) of resident peritoneal MPs and purified splenic T cells from 8 week old NOD, congenic, and control mice was detected by ELISA co-culture for 16 hours with and without a PGS-2 specific inhibitor, NS-395 (5 uM). For the NOD.B10C1 MP/T co-culture, T cells are derived from NOD mice and MPs are from NOD.B10C1 congenic mice. Values are given as the mean ± SEM. NOD, n=4 experiments; BALB/c, n=4; C57BL/6, n=4; NOD.B10C1, n=3. One-way ANOVA was used to compare the means of PGE-2 production in different MPs, p= 0.06.

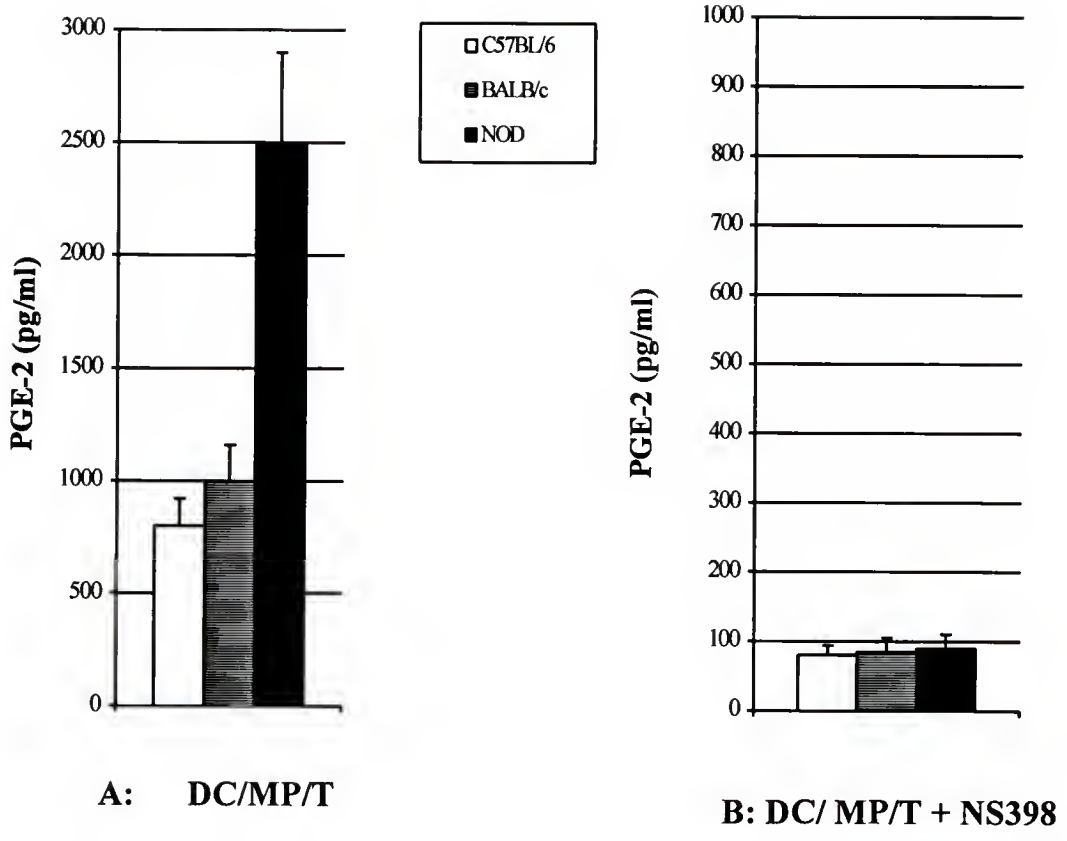


Figure 12. PGE-2 production by dendritic cell, murine macrophage and T cell co-culture. PGE-2 production from DC ($1x10^4$)/ MP ($1x10^5$) /T cell ($5x10^5$) co-culture (MEM media without phenol red and with 2% fetal bovine serum) of lymph node dendritic cells, resident peritoneal MPs and purified splenic T cells of 8 week old NOD, and control mice was detected by ELISA after co-culture for 16 hours with and without a PGS-2 specific inhibitor, NS-395 (5 uM). Values are given as the mean ± SEM. NOD, n=4 experiments; BALB/c, n=4; C57BL/6, n=4. One-way ANOVA was used to compare the means of PGE-2 production, $p< 0.04$.

(Invitrogen) and sequencing. She found no sequence differences between NOD, C57BL/6, and published data.

Sex Hormones Influence on PGS-2 Gene Expression in the NOD Mice

The sexual dimorphism in the incidence of diabetes suggests that sex hormones may play a role in the autoimmunity of the NOD mouse. The influence of sex steroid hormones has been demonstrated by exacerbation of disease in orchiectomized or estrogen treated NOD male mice, and a reduction in insulitis and diabetes incidence following oopherectomy or treatment with androgens in female mice (Fox, 1992; Hawkins et al., 1993; Fitzpatrick et al., 1991). Since estrogen and progesterone receptors are expressed on macrophage and female sex steroid hormones have been described to stimulate PGE-2 production (Smith et al., 1986; Yagel et al., 1987), these hormones may influence prostanoid metabolism through the induction of PGS-2. The murine female estrus cycle lasts about 4-5 days and the estrus phase about 12 hours; with estrogen and progesterone reaching their highest level during estrus phase (Allen, 1922). I therefore hypothesized that estrus related changes in sex steroids may play a role in the regulation of PGS-2 mRNA and protein expression. My data demonstrate a sexual dimorphism in the expression of PGS-2 in female NOD mice and dependence on the estrus phase. I found first, that the level of peritoneal MP PGS-2 mRNA expression was lower in female mice during non-estrus phase compared with that of estrus phase (table 3). Second, PGS-2 protein expression also varies during the estrus cycle as the protein was only detected during estrus phase (table 3). Third, PGS-2 protein expression in NOD female mice was only detected in sexually mature mice as it was expressed at 4 week old but not in

sexually immature 2 week old female NOD mice (table 5). Finally, PGS-2 mRNA (figure 13) and protein (figure 14) expression were not found in male NOD MPs.

In mice, PGE-2 production from macrophages has been reported to be upregulated by exposure to 10^{-7} to 10^{-8} M concentrations of progesterone (Smith et al., 1986; Yagel et al., 1987). The mechanism for this enhancement is still unknown. I hypothesized that sex hormones may affect PGE-2 production through regulation of PGS-2 expression. Sex hormone regulation of PGS-2 expression was examined in both *in vitro* and *in vivo* experiments to test this hypothesis.

I demonstrated that physiological concentrations of estradiol (10^{-6} M) and progesterone (10^{-7}) upregulate PGS-2 protein expression in NOD non-estrus female and male NOD MPs (figure 15). In contrast, the male hormones testosterone (10^{-7} M), has no effect on the PGS-2 mRNA and protein expression in NOD female MPs (figure 15). I also castrated 8 week old male NOD mice and examined PGS-2 expression four weeks after orchiectomy and found that PGS-2 mRNA and protein were not affected by removal of the testicles (table 4). PGS-2 mRNA and protein expression in female and male resident peritoneal MPs are summarized in table 3 and table 4. Table 5 summarizes PGS-2 expression in NOD female mice of different ages.

Role of Monokines on the Regulation of PGS-2 Expression in NOD Mice

PGS-2 expression in MPs can be induced by the monokines TNF-α and IL-1 (Ristimaki et al., 1994; Lee et al., 1992). Once expressed, PGE-2 production can suppress TNF-α and IL-1 gene expression while up-regulating IL-10 expression. IL-10 production, in turn, suppresses TNF-α, IL-1, and PGS-2 expression (Mertz et al., 1994). In addition

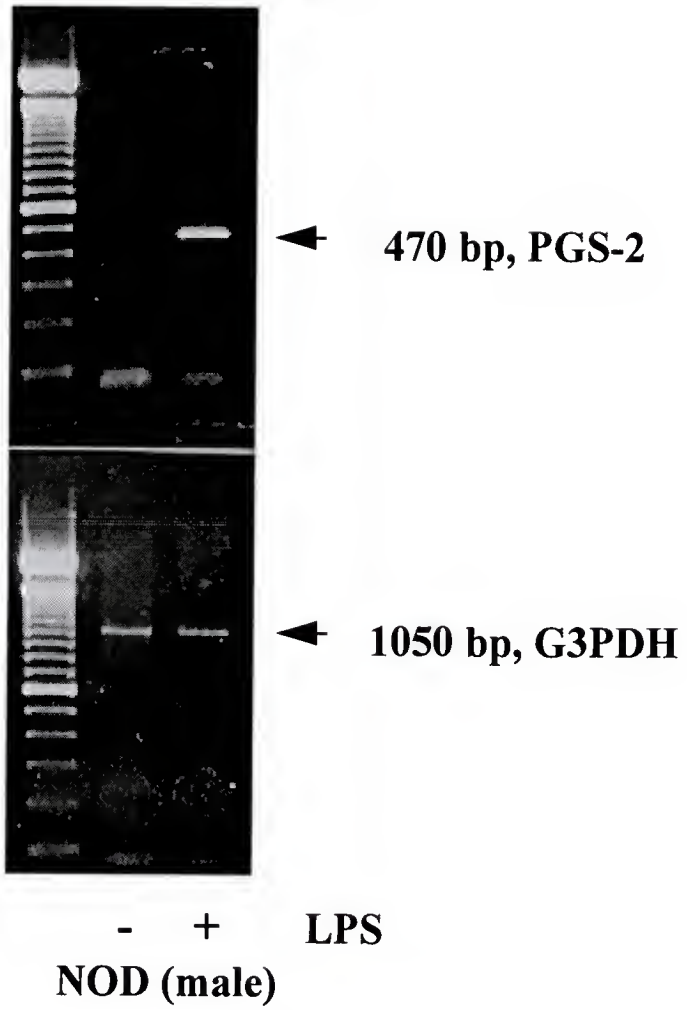


Figure 13. PGS-2 mRNA expression in male NOD mice. mRNA expression was assessed in 1 x 10^5 resident peritoneal MPs from 8 week old male mice by RT-PCR after overnight culture with and without LPS stimulation. The male NOD mice do not express, or express limited amounts of PGS-2 mRNA. PGS-2 is readily induced by LPS (10ug/ml).

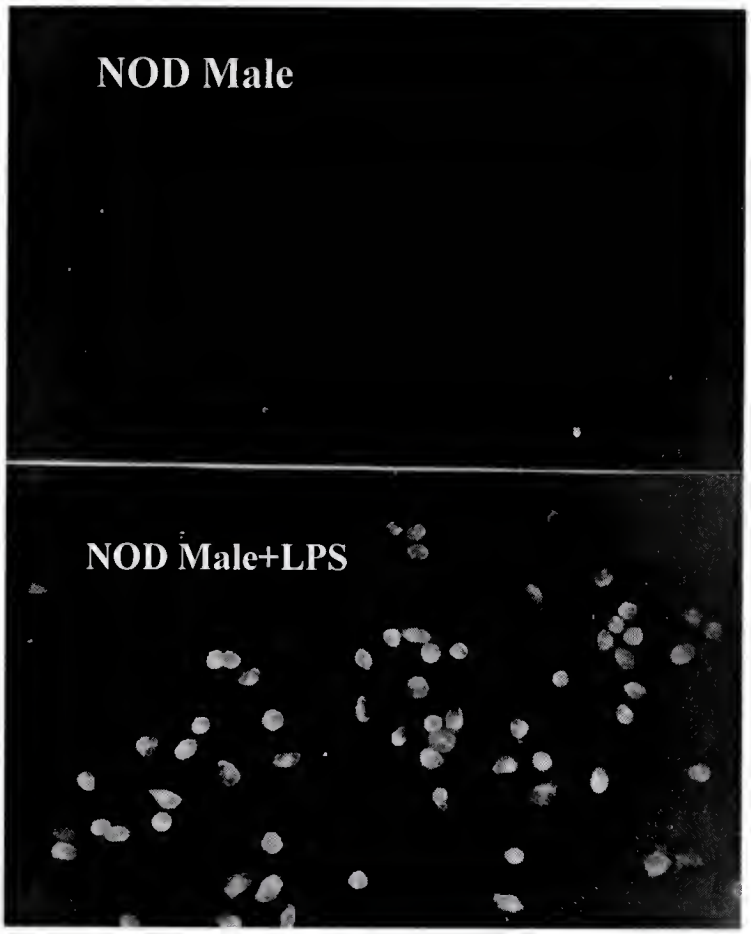


Figure 14. Expression of PGS-2 protein in resident peritoneal MPs of 8 week old NOD male mice was detected by indirect immunofluorescence using PGS-2 specific anti-sera (Reddy et al., 1994) after MPs were cultured for 16 hours with or without LPS stimulation (10 ug/ml). NOD male MPs did not spontaneously express PGS-2 protein, but it was readily induced by LPS stimulation.

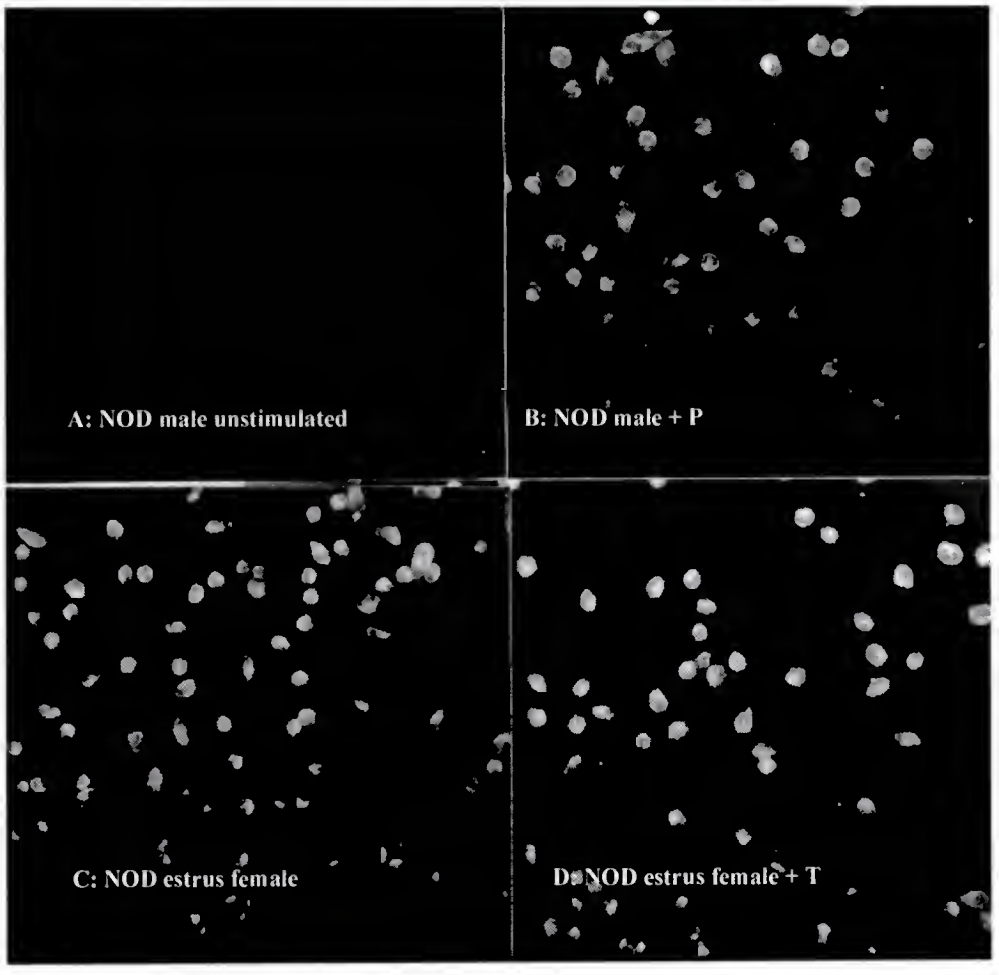


Figure 15. Effect of sex steroid hormones on the expression of PGS-2 protein in resident peritoneal MPs of 8 week old NOD (male and female). PGS-2 was detected by indirect immunofluorescence using PGS-2 specific anti-sera after MPs were cultured for 16 hours with or without stimulation. NOD male PGS-2 expression (A) is negative as is NOD non-estrus female MP expression, but can be induced by progesterone (P, 10^{-7} M) (B). NOD estrus female MPs spontaneously express PGS-2 protein (C) and testosterone (T, 10^{-8} M) had no effect on PGS-2 expression in estrus female NOD MPs (D).

Table 3. Summary of PGS-2 mRNA and protein expression in female resident peritoneal MPs. mRNA and protein expression were assessed in resident peritoneal MPs from 8 week old mice (estrus and non-estrus phase) by RT-PCR and immunocytochemistry after overnight culture with or without LPS (10 ug/ml), and with or without various sex steroid hormones at physiological concentration (NOD only). Estradiol (10^{-6} M), progesterone (10^{-7} M), and testosterone (10^{-7} M).

	PGS-2 mRNA (Estrus)	PGS-2 mRNA (Non-Estrus)	PGS-2 Protein (Estrus)	PGS-2 Protein (Non-Estrus)
C57BL/6	-	-	-	-
BALB/c	-	-	-	-
NOD	+	+	+	-/low
NOD scid/scid	+	+	+	-/low
C57BL/6+LPS	+	+	+	+
BALB/c + LPS	+	+	+	+
NOD + LPS	+	+	+	+
NODscid/scid+LPS	+	+	+	+
NOD + Estradiol	+	+	+	+
NOD + Progesterone	+	+	+	+
NOD + Testosterone	+	+	+	-/low

Table 4. Summary of PGS-2 mRNA and protein expression in male resident peritoneal MPs. mRNA and protein expression were assessed in resident peritoneal MPs from 8 weeks old mice by RT-PCR and immunocytochemistry assay after overnight culture with or without LPS, and without or with estradiol (10^{-6} M) and progesterone (10^{-7} M). NOD males were also castrated at 8 weeks of age. Four weeks after castration, mRNA and protein expression was assessed in peritoneal MPs after overnight culture.

	PGS-2 mRNA	PGS-2 Protein
BALB/c	-	-
BALB/c + LPS	+	+
NOD	-	-
NOD + LPS	+	+
Castrated NOD	-	-
NOD + Estradiol	+	+
NOD + Progesterone	+	+

Table 5. Summary of PGS-2 protein expression in female resident peritoneal MPs. PGS-2 protein expression was assessed in resident peritoneal MPs from mice by immunocytochemistry after overnight culture.

	2 Wks	4 Wks	6 Wks	8 Wks	12 Wks	16 Wks	20 Wks
PGS-2	-	+	+	+	+	+	+

to monokines, cytokines produced by T cells, i.e., IFN-γ, upregulate PGS-2 expression, and both T cell and MP derived TGF-β can suppress PGS-2 expression (Reddy et al., 1993).

The spontaneous expression of PGS-2 in NOD could be due to: 1) a primary defect in regulatory elements of the PGS-2 gene, or 2) a defect in the monokines that affect PGS-2 expression. Studies have shown that TNF-α and IL-1 can upregulate PGS-2 expression. Defects leading to enhanced production of TNF-α or IL-1 may ultimately contribute to constitutive expression of PGS-2 in the NOD. On the other hand, IL-10 or TGF-β can suppress PGS-2 expression, so defects in the down regulation of PGS-2 by IL-10 or TGF-β could also contribute to the differential expression of PGS-2 in NOD MPs. Therefore, perturbing the autocrine regulation of PGS-2 may contribute to the spontaneous expression of PGS-2 in NOD MPs.

To investigate the influence of monokines on PGS-2 expression, TNF-α, and IL-1β production were quantitated from the supernatant of $1x10^5$ resident peritoneal MPs of NOD, BALB/c and C57BL/6 mice cultured overnight. The production of monokines were found to be undetectable by ELISA (assay detection limit 30 pg/ml and 70 pg/ml respectively) in all three mouse strains examined; and were produced at similar levels following LPS stimulation (about 70 pg/ml for TNF-α, and 130 pg/ml for IL-1β). These data suggest that spontaneous PGS-2 expression is not due to the enhanced production of the monokines TNF-α and IL-1β by NOD MPs. Since the TNF-α and IL-1 levels in MPs cell culture are below ELISA detection limit, further *in vitro* studies to investigate the effect of quantitative TNF-α and IL-1 neutralizing antibodies on the NOD MPs PGS-2

expression can more directly address the issue of the sensitivity of PGS-2 expression to the autocrine TNF-α and IL-1 in NOD MPs.

Previous studies have shown that PGS-2 mRNA and protein expression is readily suppressed by IL-10 (Mertz et al., 1994). If IL-10 production in NOD MPs is absent or low, or if PGS-2 becomes insensitive to IL-10 regulation in NOD MPs, then, PGS-2 expression may be enhanced. IL-10 production from the supernatant of $1x10^5$ resident peritoneal MPs of both BALB/c and NOD mice cultured overnight was examined. IL-10 in these samples was undetectable (below 30 pg/ml detection limit) but can be induced by LPS to similar levels (NOD:70 pg/ml Vs BALB/c: 75 pg/ml). These data suggest that following LPS stimulation, NOD mice produce IL-10 at the levels which normally would suppress PGS-2 expression. To examine the regulation of PGS-2 by IL-10, I examined the suppressive effect of this cytokine on PGS-2 protein expression in both NOD MPs and LPS stimulated BALB/c and C57BL/6 MPs. I found that 10 and 100 ng/ml rmIL-10 totally blocked the LPS induced PGS-2 expression in BALB/c and C57BL/6 mice (figure 16). In contrast, up to 500 ng/ml rmIL-10 did not block spontaneous PGS-2 expression in NOD MPs. These data suggest that there may be a general desensitization in NOD MP responses to IL-10 which could be due to a defect in the IL-10 receptor or post-IL-10 receptor signal transduction cascade. This data may also provide a mechanism for spontaneous expression of PGS-2 in NOD MP: the loss of the suppression function of IL-10. Alternatively, there may be a differential regulation regarding IL-10 mediated suppression of PGS-2 expression. i.e., LPS induced PGS-2 expression is sensitive to IL-10 suppression; whereas, spontaneous PGS-2 expression in NOD mice which is

upregulated by sex steroid hormones may be IL-10 insensitive. Further experiments are needed to elucidate the mechanisms responsible for the defect in the IL-10 suppression of PGS-2 expression in NOD MPs. Table 6 summarizes the rmIL-10 inhibitory effect on PGS-2 protein expression.

Contribution of Spontaneous PGS-2 to the Immunopathogenesis of IDD, a Candidate Gene for *Idd5*

A particularly powerful approach to the study of polygenetic disease is the development of congenic mouse strains with genetically defined segments of chromosomes from non-autoimmune control mice on the background of the autoimmune mouse, or visa versa. Once established, these strains can be used to assess the effect of these chromosomal segments on disease or on an immunophenotype, i.e., PGS-2 expression in our case. The immunophenotype may be particularly helpful in suggesting candidate genes in the congenic interval if sufficiently well characterized.

My studies suggest the constitutive expression of PGS-2 is a primary defect based on data from NODscid/scid mice. I hypothesize that aberrant PGS-2 expression may be genetically determined. I, hence, examined PGS-2 expression in congenic mouse strains that contain segments of chromosome 1 which include the PGS-2 gene from either NOD or C57BL/10snj. The first congenic strain examined was NOD.B10C1. This strain was derived by Dr. Linda Wicker's laboratory at Merck Laboratory, Rahway, NJ. These mice contain an interval of C57BL/10snj chromosome 1 that includes the B10 PGS-2 gene on the NOD background (figure 17). In female homozygous NOD.B10C1 mice, replacement of the NOD chromosome 1 segment with the C57BL/10snj segment delayed the onset of diabetes to 14 weeks of age (compared with onset in NOD which is 10 weeks of age) and

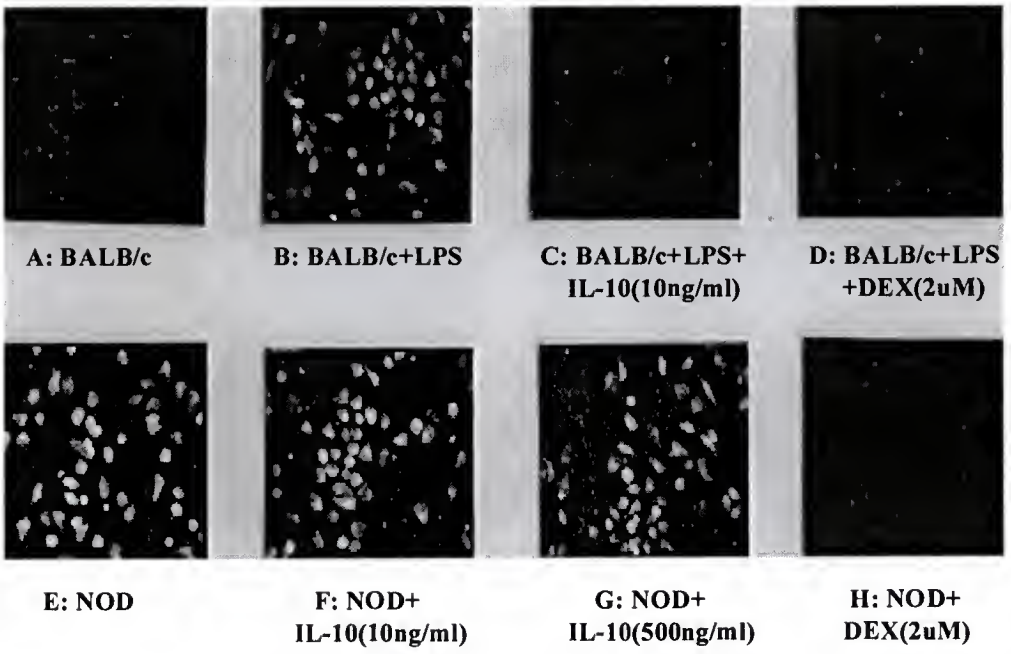


Figure 16. mrIL-10 suppresses LPS induced PGS-2 expression in BALB/c but not spontaneously expressed PGS-2 in estrus NOD mice. Peritoneal MPs ($1x10^5$) were cultured with or without LPS (10 ug/ml) and/or different doses of mrIL-10 overnight. NOD PGS-2 protein expression is completely suppressed by dexamethasone (DEX, 2 uM). PGS-2 protein was examined by immufluorescence detection using a PGS-2 specific anti-sera (Reddy et al., 1994).

Table 6. Summary of rmIL-10 mediated suppression of PGS-2 protein expression in resident peritoneal MPs of female mice. Protein expression was assessed in resident peritoneal MPs from 8 week old female mice (estrus phase) by immunocytochemistry after overnight culture with or without LPS (10 ug/ml), in combination with dexamethasone (DEX, 2 uM) and mrIL-10 (10, 100, and 500 ng/ml).

	BALB/c	C57BL/6	NOD
MPs	—	—	++
MPs+IL-10 (100 ng/ml)	—	—	++
MPs+IL-10 (500 ng/ml)	—	—	++
MPs+DEX	—	—	—
MPs+LPS	++	++	++
MPs+LPS+DEX (2 uM)	—	—	—
MPs+LPS+IL-10 (10 ng/ml)	—	—	++
MPs+LPS+IL-10 (500 ng/ml)	—	—	++

the cumulative incidence of diabetes by the age of 30 weeks was decreased to approximately 40% (compared with NOD which is 80%) (figure 18). I examined PGS-2 expression in the resident peritoneal MPs of estrus female NOD.B10C1 mice and found that MPs from NOD.B10C1, unlike those from parental NOD mice, do not express PGS-2 mRNA or protein (figure 19). As a control, PGS-2 protein expression in NOD.B10C3 (C57BL/10snj chromosome 3) and NOD.B10C4 (C57BL/10snj chromosome 4) congenic mice were also examined. Both of these congenic strains have the NOD PGS-2 gene and express the PGS-2 protein to the same level as the NOD MPs (figure 20)

I also examined the B6.NODC1 congenic mouse. The B6.NODC1 mouse was derived in Dr. Edward Wakeland's laboratory (University of Florida, Gainesville, FL) and contains an interval of NOD chromosome 1 (*Idd 5*) including NOD PGS-2 on the C57BL/6 background (figure 17). B6.NODC1 develop peri-insulitis by 6 months of age. I found that MPs from estrus B6.NODC1 mice constitutively express PGS-2 protein (figure 19). PGS-2 expression in these mice was further confirmed by Western blotting (figure 20).

My studies demonstrated that the PGS-2 phenotype is correlated with the autoimmune phenotype in these congenic animals. The positive expression of PGS-2 in B6.NODC1 mice is correlated with the development of peri-insulitis in this congenic strain; whereas, the lack of expression of PGS-2 in NOD.B10C1 is correlated with a 50% drop of diabetes incidence by 30 weeks of age. These data suggest that PGS-2 is a candidate susceptibility gene for *Idd5* in NOD mice.

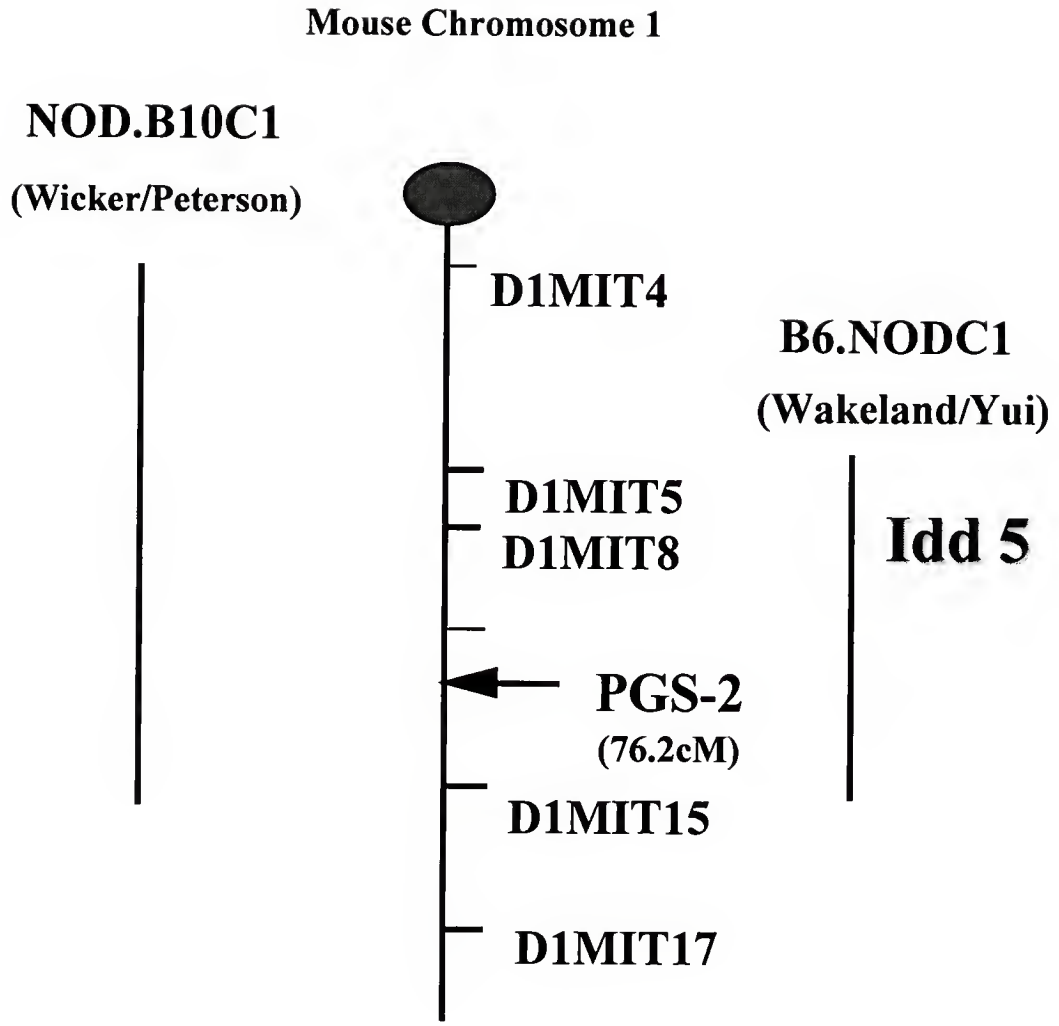


Figure 17. Location of the PGS-2 gene on chromosome 1 and the intervals included in the congenic mouse strains evaluated.

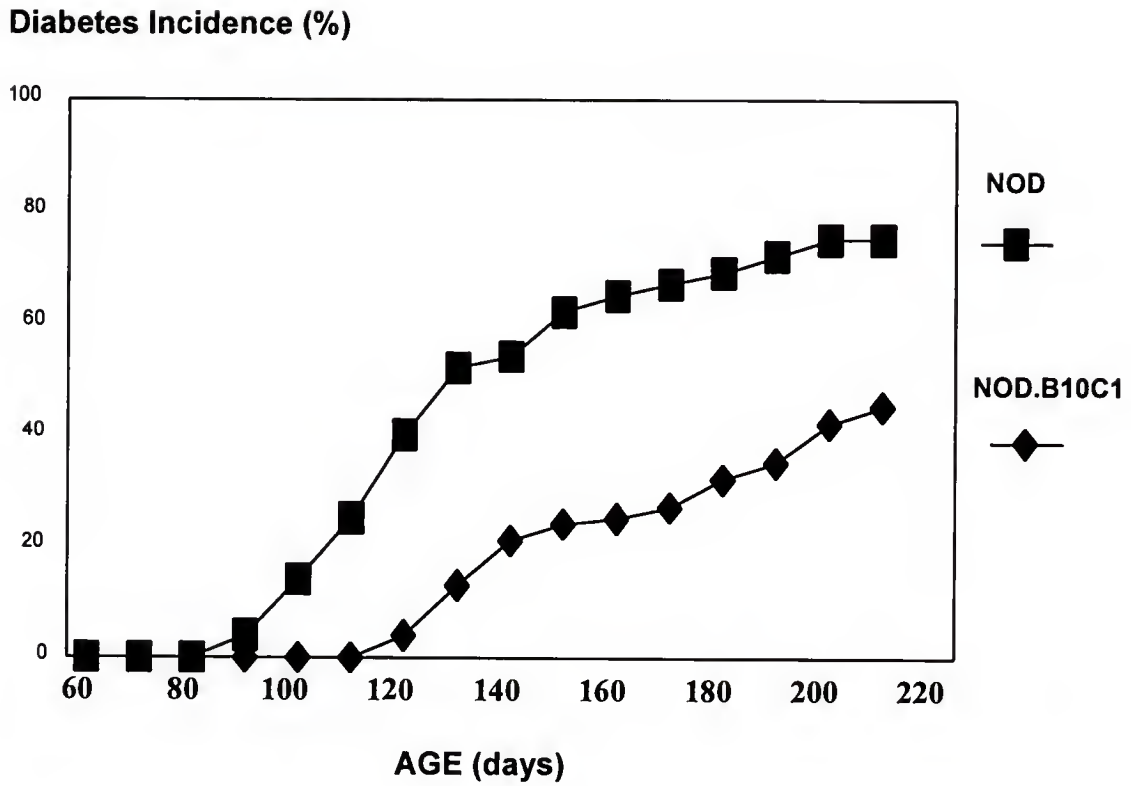


Figure 18. Cumulative incidence of diabetes in NOD.B10C1 congenic mice (from Dr. Linder Wicker, Merck Laboratory, Rahway, NJ).

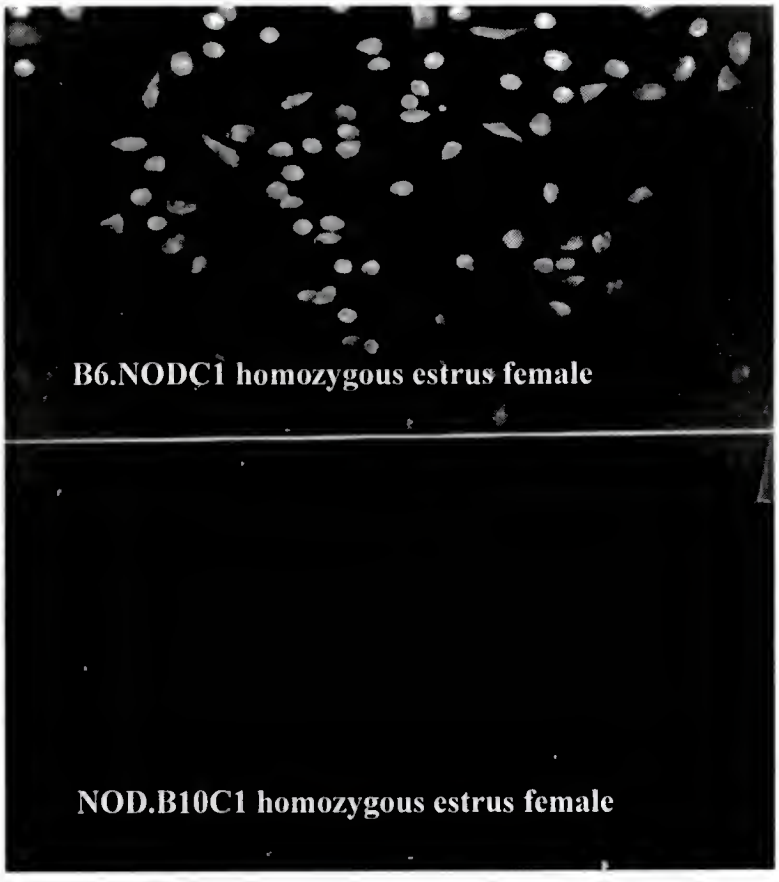


Figure 19. Expression of PGS-2 protein in resident peritoneal MPs of 8 week old female estrus congenic mice NOD.B10C1 and B6.NODC1. PGS-2 was detected by indirect immunofluorescence using a specific PGS-2 specific anti-sera (Reddy et al., 1994) after MPs were cultured for 16 hours without stimulation. Both homozygous and heterozygous (not shown) B6.NODC1 spontaneously express PGS-2, while NOD.B10C1 homozygous mice do not.

Table 7. Summary of background information and MP PGS-2 expression in the congenic strains NOD.B10C1 and B6.NODC1.

	NOD.B10C1	B6.NODC1
Background	NOD	C57BL/10snj
Chrom.1/PGS-2	C57BL/10snj	NOD
Insulitis/ Diabetes Incidence	Insulitis/ Diabetes Incidence: 45%	Peri-Insulitis/ No Diabetes
PGS-2 Expression	-	+

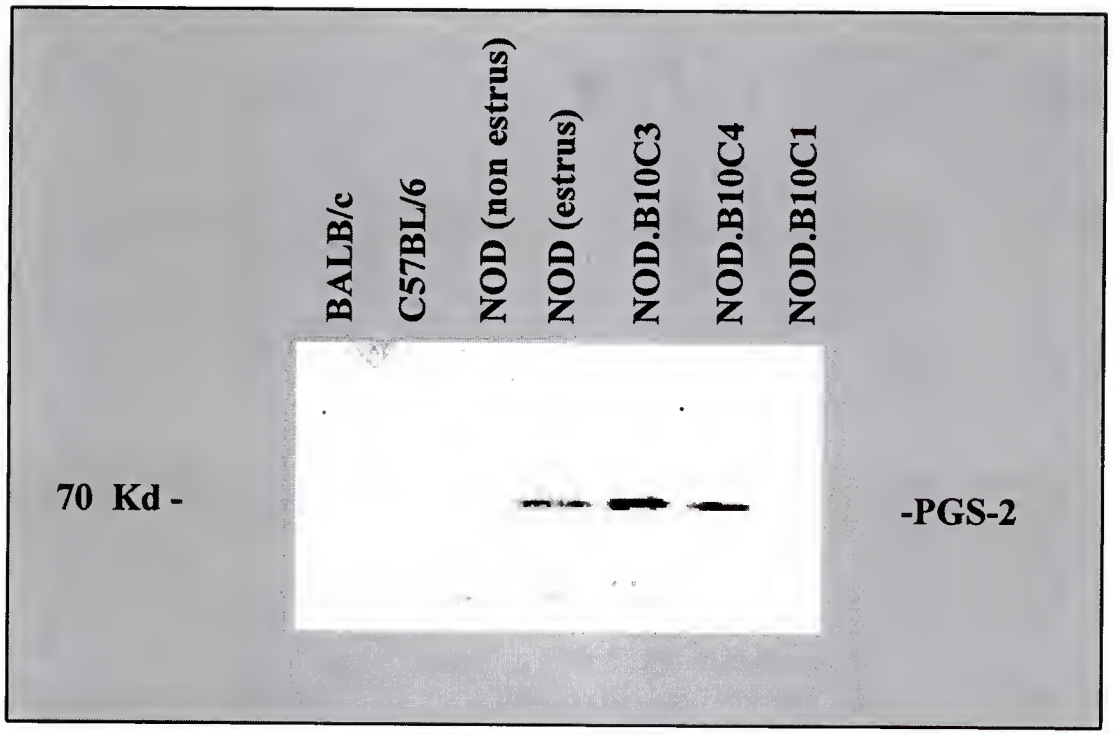


Figure 20. Detection of PGS-2 protein in resident peritoneal MPs from 8 week old female estrus NOD, non estrous NOD, NOD.B10C1, NOD.B10C3, NOD.B10C4, and control mouse strains. PGS-2 was detected by SDS-polyacrylamide gel electrophoresis (5% stacking and 8% resolving gel) and Western blotting. Fresh resident peritoneal MPs ($2x10^6$) were harvested in SDS gel loading buffer. Extracts were prepared and subjected to electrophoresis and western analysis with PGS-2 specific antiserum (Reddy et al., 1994). Seventy five ug of protein was loaded per lane.

Table 8. Summary of PGS-2 expression in NOD, NODscid/scid, congenic, and parental mouse strains.

	Background	Chrom. 1	PGS-2	Insulitis/Diabetes Mellitus
NOD	NOD	NOD	+	+/80%
NODscid/scid	NOD	NOD	+	-/0%
B6.NODC1	C57BL/6	NOD	+	Peri-insulitis/0%
C57BL/6	C57BL/6	C57BL/6	-	-/0%
NOD.B10C1	NOD	C57BL/10snj	-	+/45%
C57BL/10snj	C57BL/10snj	C57BL/10snj	-	-/0%

PGS and iNOS Inhibitors Affect NOD Diabetes Incidence

Our data suggest that aberrantly expressed PGS-2 contributes to the enhanced PGE-2 production in NOD MPs. Since PGE-2 has multiple important effects on the immune response and peripheral tolerance, we investigated whether blocking PGS-2 enzyme activity pharmacologically prevents diabetes in NOD. We treated NOD mice with a combination of indomethacin, a PGS-2 inhibitor, and aminoguanidine, an inducible nitric oxide synthase (iNOS) inhibitor. We chose this combination because our data suggested that NO also contributes to MP mediated suppression of T cell activation, and because NO markedly enhances the enzymatic activity of PGS-2 (Salvemini et al., 1993; Franchi et al., 1994). In two separate experiments, we either treated NOD female mice from 8 weeks of age with a combination of indomethacin (3 ug/ml) and aminoguanidine (0.1%) or from 4 weeks of age with high doses of indomethacin alone (20 ug/ml) in the drinking water. In an initial study, we found that treatment of NOD female mice from 8 weeks of age with a combination of indomethacin/aminoguanidine (I/A) significantly delayed the onset (onset began at 20 weeks of age in I/A treated group compared with 8 weeks in the untreated group) and reduced the diabetes incidence by 40% compared to untreated NOD of the same age. ($P < 0.02$, figure 21). In a truncated experiment, we observed that treatment of NOD female mice from 4 weeks of age with high dose of indomethacin alone (20 ug/ml) provided an identical effect in delaying the onset of diabetes and reducing the diabetes incidence and difference is also significant ($p < 0.04$, figure 22). These data further support a critical role for PGE-2 in the pathogenesis of diabetes.

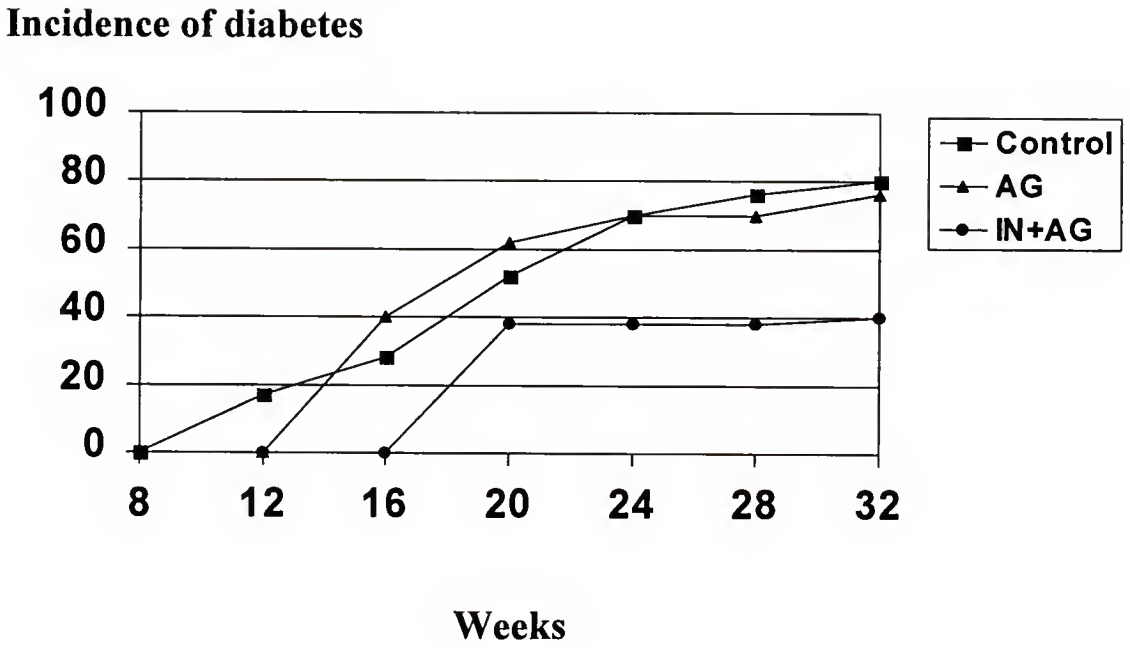


Figure 21. Cumulative incidence of diabetes in NOD mice treated with indomethacin (IN, 3 ug/ml) and aminoguanidine (AG, 0.1%). NOD female mice were treated from 8 to 32 weeks of age with indomethacin and aminoguanidine in their drinking water. Fresh drugs were added to the water during weekly water change. Diabetes was monitored weekly by a urine glucose test and confirmed by tail vain blood glucose level (250 mg/dl glucose was used as indication of diabetes). The logrank chi-square statistic (Mantel, 1988) was used to compare incidence of diabetes between the control and the IN/AG treated group. $p < 0.02$, p values are one sided. n=20 animals per group.

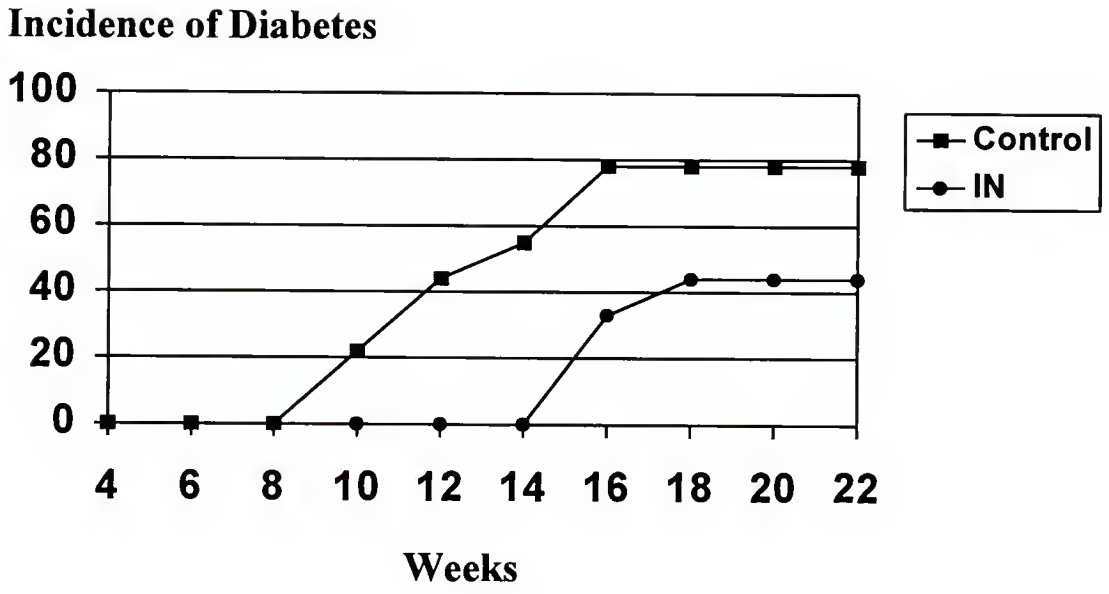


Figure 22. Cumulative incidence of diabetes in NOD mice treated with indomethacin (IN, 20 ug/ml). NOD female mice were treated from 4 to 22 weeks of age with indomethacin in their drinking water. Fresh drugs were added to the water during weekly water change. Diabetes was monitored weekly by a urine glucose test and confirmed by tail vain blood glucose level (250 mg/dl glucose was used as indication of diabetes). The logrank chi-square statistic (Mantel, 1988) was used to compare incidence of diabetes between the control and the IN/AG treated group. $p < 0.02$, p values are one sided. n=9 animals per group.

Impaired AICD in NOD Mice Secondary to Enhanced PG Production

Activation induced cell death (AICD), the apoptosis of T cells following the activation with antigen (Rocha et al., 1991; Zhang et al., 1992), is an important mechanism for peripheral T cell tolerance. Several studies have suggested that AICD can be markedly inhibited by anti-IL-2 antibodies and by agents that block the cell cycle, i.e., cAMP. Of interest, PGE-2 is a potent inhibitor of IL-2 and IL-2 receptor expression and blocks the cell cycle through its activation of adenylate cyclase and generation of cAMP (Goetzl et al., 1995; Ucker et al., 1994). Based on my data and other published studies (Lety et al., 1992) there is enhanced PG production in NOD mice. I therefore hypothesized that enhanced PGs production may result in an impaired AICD and contribute to NOD diabetes. To test my hypothesis, I followed an established *in vivo* experimental model of ACID, the immunization of mice with the bacterial superantigen, *Staphylococcus aureus enterotoxin B* (SEB), as described by MacDonald et al. (1991) and Kawabe et al. (1991). Following MacDonald's protocol, I examined ACID in the groups of untreated NOD mice, indomethacin (20ug/ml)/aminoguanidine (0.1%) (IN/AG) treated NOD and BALB/c mice (IN/AG were added to the drinking water 2 weeks prior to SEB immunization and continued on these drugs throughout the remainder of the experiment) and untreated control strains of mice (C57BL/6 and BALB/c). On day zero, animals were given an intraperitoneal injection of buffered saline or SEB. Splenic cells from three mice in each group were examined on day 2 and day 10 after immunization for the percentage of Vβ8+/CD4+ T cells and Vβ6+/CD4+ T cells by fluorescence activated cell sorter (FACS) analysis (figure 23, 24, 25).

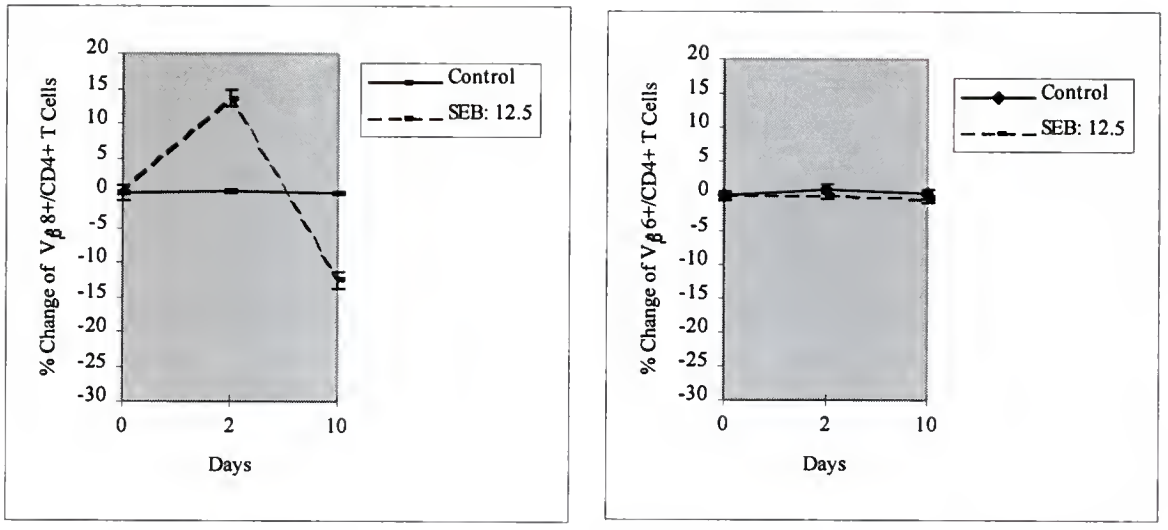
In addition to the effects of PGS-2, MHC molecules may also affect AICD in the NOD mouse. MHC molecules play a crucial role in immune response. Any defect in MHC molecules leading to down regulation of antigen presentation or activation of T cell would directly impair immune tolerance. Changes or variations in MHC molecules are associated with several autoimmune diseases (Erlich et al., 1993). Studies also demonstrated that the unique H-2^{g7} MHC plays a central role in NOD autoimmunity (Hattori et al., 1986; Prochazka et al., 1987; Ikegami et al., 1988). Recent studies suggest that the NOD MHC molecule is inherently unstable and as it does not readily dimerize, that decreases its efficiency in binding and presenting antigens (Carrasco-Marin et al., 1996). I therefore hypothesized that unique properties of H-2^{g7} in combination with aberrant PGS-2 expression may disturb its ability to present self antigen to self reactive T cells and impair AICD in NOD mice.

AICD in NOD.H-2^{b} congenic mice were examined as control for the effect of NOD MHC molecule (figure 26). These mice express the C57BL/6 H-2^{b} MHC gene on a NOD background.

Figure 23 shows the expansion and deletion of Vβ8+/CD4+ T cells in response to SEB (12.5 ug) immunization in C57BL/6 mice. BALB/c mice were also immunized with SEB (50 ug) and a similar pattern of expansion and deletion as that of C57BL/6 was seen but at higher levels (figure 24). Figure 25 illustrates the markedly impaired expansion of the Vβ8+ T cell population in NOD. However, if NOD mice are pre-treated with drugs that block PGS-2 activity, the early expansion is not affected, but deletion on day 10 increased two fold. Similar indomethacin and aminoguanidine treatment of BALB/c mice

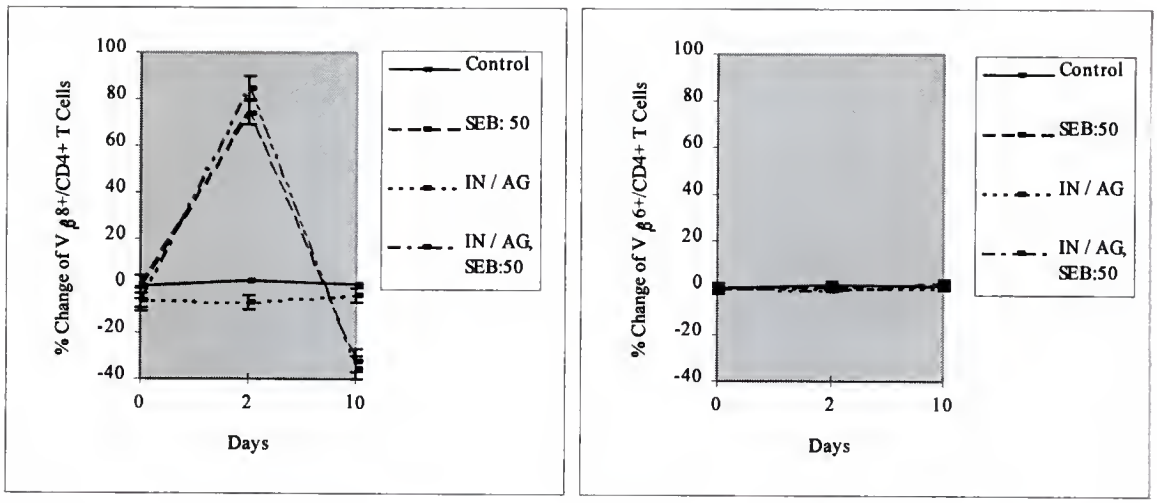
did not shown effect on the early expansion and later deletion of Vβ8+/CD4+ T cells (figure 24). The potent effect of the NOD MHC H-2^{g7} on the expansion and deletion of Vβ8+ T cells is illustrated in the data shown in figure 26.

These experiments demonstrated that AICD in NOD is predominantly affected by the MHC molecule and that PGS-2 contributes, but to a much lesser degree than MHC to the impairment of deletion. Our study suggests that the NOD MHC molecule incombination with PGS-2 expression plays a dramatic role in the impairment of AICD in superantigen driven expansion and deletion of T cells in NOD mouse.



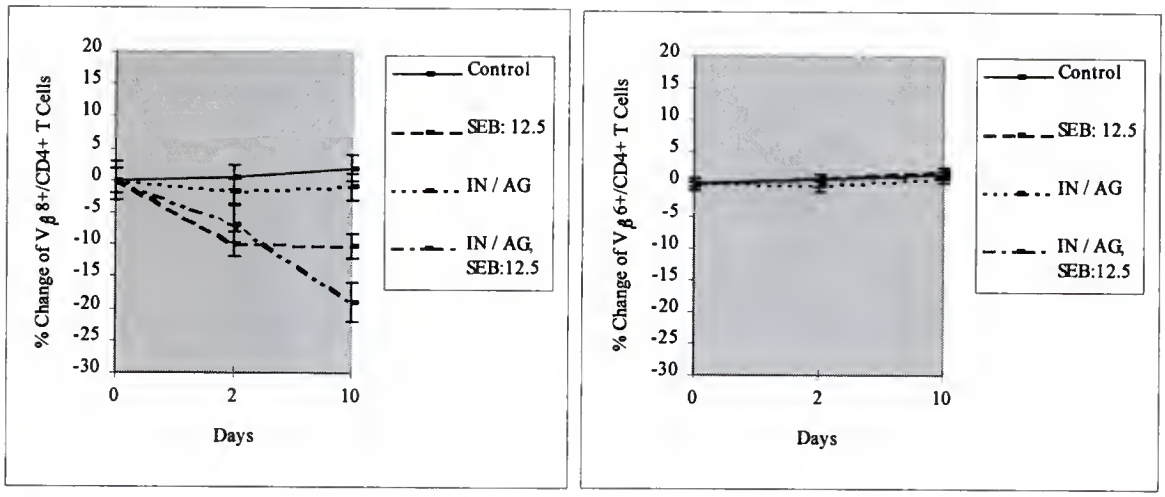
a. b.

Figure 23. Percent change in Vβ8+/CD4+ and Vβ6+/CD4+ splenic T cells in SEB (12.5 ug) immunized C57BL/6 mice. Mice were immunized with 12.5 ug of SEB by intraperitoneal injection and the number of splenic Vβ8+/CD4+ (a) and Vβ6+/CD4+ (b) were measured by dual color FACS analysis on day 2 and day 10 following immunization. Three animals at each time point were tested. Results are expressed as mean value of percentage change ± SEM (Percent change is the percent of Vβ8+/CD4+ or Vβ6+/CD4+ T cells in each individual animal subtract the mean value of percent of Vβ8+/CD4+ or Vβ6+/CD4+ T cells in control group, and then divided by the mean value of percent of Vβ8+/CD4+ or Vβ6+/CD4+ T cells in control group).



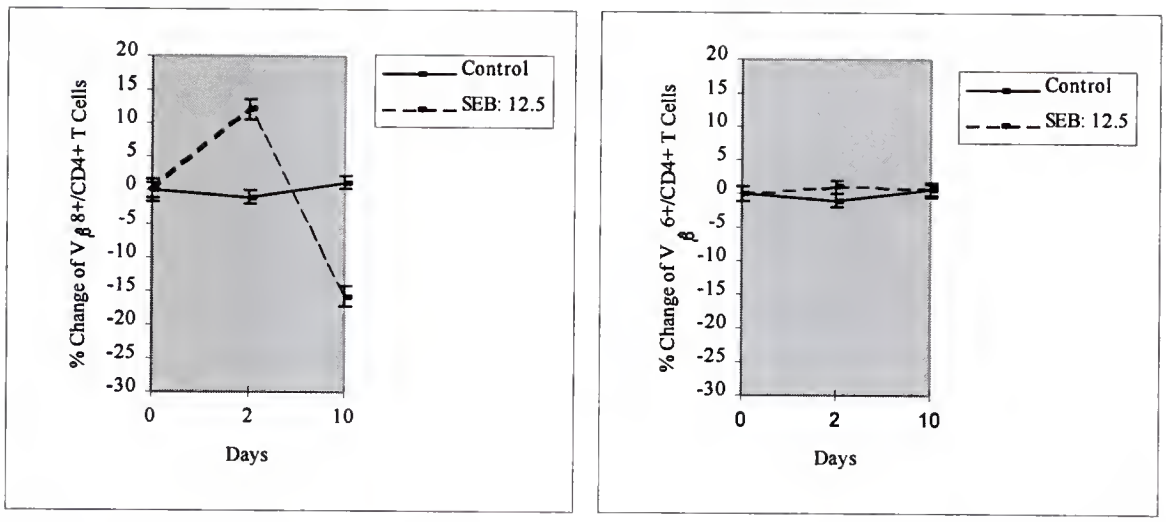
a. b.

Figure 24. Percent change in Vβ8+/CD4+ and Vβ6+/CD4+ splenic T cells in SEB (50 ug) immunized BALB/c mice. Untreated BALB/c mice and BALB/c mice treated for 2 weeks with indomethacin (IN, 20 ug/ml)/aminoguanidine (AG, 0.1%) were immunized with 50 ug of SEB by intraperitoneal injection and the number of splenic Vβ8+/CD4+ (a) and Vβ6+/CD4+ (b) were measured by dual color FACS analysis on day 2 and day 10 following immunization. Three animals at each time point were tested. Results are expressed as mean value of percentage change ± SEM (Percent change is the percent of Vβ8+/CD4+ or Vβ6+/CD4+ T cells in each individual animal subtract the mean value of percent of Vβ8+/CD4+ or Vβ6+/CD4+ T cells in control group, and then divided by the mean value of percent of Vβ8+/CD4+ or Vβ6+/CD4+ T cells in control group).



a. b.

Figure 25. Percent change in Vβ8+/CD4+ and Vβ6+/CD4+ splenic T cells in SEB (12.5 ug) immunized NOD mice. Untreated NOD mice and NOD mice treated for 2 weeks with indomethacin (IN, 20 ug/ml)\aminoguanidine (AG, 0.1%) were immunized with 12.5 ug of SEB by intraperitoneal injection and the number of splenic Vβ8+/CD4+ (a) and Vβ6+/CD4+ (b) were measured by dual color FACS analysis on day 2 and day 10 following immunization. Three animals at each time point were tested. Results are expressed as mean value of percentage change ± SEM (Percent change is the percent of Vβ8+/CD4+ or Vβ6+/CD4+ T cells in each individual animal subtract the mean value of percent of Vβ8+/CD4+ or Vβ6+/CD4+ T cells in control group, and then divided by the mean value of percent of Vβ8+/CD4+ or Vβ6+/CD4+ T cells in control group).



a. b.

Figure 26. Percent change in Vβ8+/CD4+ and Vβ6+/CD4+ splenic T cells in SEB (12.5 ug) immunized NOD.H-2^b congenic mice. Mice were immunized with 12.5 ug of SEB by intraperitoneal injection and the number of splenic Vβ8+/CD4+ (a) and Vβ6+/CD4+ (b) were measured by dual color FACS analysis on day 2 and day 10 following immunization. Three animals at each time point were tested. Results are expressed as mean value of percentage change ± SEM (Percent change is the percent of Vβ8+/CD4+ or Vβ6+/CD4+ T cells in each individual animal subtract the mean value of percent of Vβ8+/CD4+ or Vβ6+/CD4+ T cells in control group, and then divided by the mean value of percent of Vβ8+/CD4+ or Vβ6+/CD4+ T cells in control group).

CHAPTER 4
DISCUSSION

Insulin-dependent diabetes (IDD) is an autoimmune disease. The non-obese diabetic mouse is a well established, spontaneous animal model of the human disease (Makino et al., 1980). Both T cells and antigen presenting cells, especially macrophages, appear to play important roles in the immunopathogenesis of this disease. Several studies have suggested that antigen presenting cells are defective in their capacity to active T cells in IDD (Yokona et al., 1989; Ransanen et al., 1989; Rapoport et al., 1993; Serrze et al., 1990). The cellular and molecular basis of this defect, however, is largely unknown. The studies presented here demonstrate that resident NOD peritoneal MPs, unlike control MPs, spontaneously express mRNA transcripts and protein for the early response gene prostaglandin synthase II (PGS-2). My initial studies suggest that PGS-2 insensitivity to the suppression by murine IL-10 may play a role in its constitutive expression. I also found that constitutive expression of PGS-2 in NOD MPs is responsible for enhanced prostanoid production, and that treating NOD mice with drugs that block PGS-2 enzymatic activity significantly delay the onset and reduce the incidence of diabetes. I further demonstrate that enhanced prostaglandin production may interfere with tolerance mechanisms by impairing AICD. Finally, my data from chromosome 1 congenic mice suggest a genetic basis for the NOD PGS-2 phenotype and its candidacy as a diabetes susceptibility gene.

My data show that unstimulated peritoneal NOD MPs from 8 week old estrus female NOD mice housed in a SPF facility constitutively express high levels of PGS-2 mRNA and protein (figure 6, 7). In contrast, unstimulated resident MPs from control strains (BALB/c, C57BL/6, C57BL/10) do not express this enzyme without induction by agents such as LPS. To exclude the possibility that the autoimmune environment and autoimmune T and B cells in NOD mice contribute to the PGS-2 expression, I also examined its expression in MPs of female NODscid/scid mouse. The NODscid/scid mouse which lacks functional T and B cells and consequently does not develop autoimmunity, also expresses PGS-2 constitutively. These data suggest that the autoimmune environment is not necessary for its expression and may be a primary defect in the regulation of PGS-2 in MP of NOD mice.

Because PGS-2 was differentially expressed in NOD mice, its expression may result in enhanced prostaglandin production by NOD MP. PGE-2 production was analyzed in MPs cell culture supernatant of NOD, NODscid/scid, and control mice. PGE-2 production of resident peritoneal MPs was found to be consistently higher than control MPs. Also PGE-2 levels produced by NODscid/scid MPs are similar to those of NOD mice. Furthermore, PGE-2 production by NOD and NODscid/scid MPs is mediated by the PGS-2 enzyme since a PGS-2 specific inhibitor, NS-398, completely blocks its production. These data demonstrated that there is an enhanced PG production in NOD MPs and that this production is secondary to the constitutive expression of PGS-2.

Since the finding indicate NOD PGS-2 mRNA is constitutively expressed and protein expression is up regulated during estrus phase, both transcriptional and translational regulation may be involved in the aberrant PGS-2 expression.

The expression of PGS-2 expression following induction is regulated mainly by transcriptional activation, but post-transcriptional regulation also occurs (DeWitt et al., 1993; Evett et al., 1993; Ristimaki et al., 1994). PGS-2 mRNA is unstable compared with PGS-1 mRNA; a feature predicated from the presence of multiple RNA instability sequence (AUUUA) in its 3'-untranslated region. PGS-2 mRNA is translated as soon as it is synthesized; therefore, the short mRNA half-life (less than 2 hrs) limits PGS-2 production post-transcriptionally (DeWitt et al., 1993). Evett et al. (1993) suggest that instability of PGS-2 protein, a relatively short half-life of 22 min in chicken embryo fibroblasts, also plays a role in the regulation of PGS-2 enzyme expression. Due to the complex regulation and the instability of PGS-2 mRNA and protein, PGS-2 expression is transiently expressed with mRNA levels peaking at 1 hr and protein levels peaking at 2 hr following induction and returning to basal levels by 4 hr and 6 hr respectively (DeWitt et al., 1993).

My data show that PGS-2 expression in NOD MPs differs from PGS-2 expression in the MPs of control strain mice. The defect that contributes to aberrant PGS-2 expression may occur at any stage of gene expression; i.e., transcription level, post-transcription level (mRNA stability), translation level, and post translation level (protein stability).

The differential PGS-2 expression in NOD MPs suggests a genetic basis for the changes in PGS-2 expression regulation. To elucidate the possible defect in the cis-regulatory elements in the PGS-2 promoter region, the PGS-2 5' promoter region was sequenced from -966 nucleotide to the transcriptional start site and found that there are no sequence difference between NOD, C57BL/6, and published data from 3T3 fibroblasts (Fletcher et al., 1992). This analysis suggests that cis-elements in the 5' region of the NOD PGS-2 gene that I examined do not contribute to aberrant PGS-2 expression.

Although in this initial analysis, there is no genetic defect was found to account for PGS-2 expression in NOD MPs, several alternative explanations remain to be examined. The mechanisms that may contribute to aberrant PGS-2 expression could be: 1) A change in the structure of PGS-2 gene itself which may affect PGS-2 protein structure and protein stability. 2) A defect in a cis-element located 5' to the region I sequenced or 3' of PGS-2 gene. 3) A trans factor from another defective gene. Both trans activating factors and cis-regulation elements could upregulate PGS-2 expression by direct induction or through the disruption of the normal suppression of PGS-2 expression in unstimulated MPs. 4) A change in the multiple RNA instability sequences in the PGS-2 3'-untranslated region may enhance the stability of PGS-2 mRNA. 5) A defect which may enhance PGS-2 translation and protein stability.

The difference in the incidence of diabetes between males and females suggests that sex hormones may play a role in the autoimmunity of NOD. The influence of sex steroid hormones on autoimmunity has been shown by exacerbation of disease in orchiectomized or estrogen treated NOD male mice, and a reduction in insulitis and

diabetes incidence following oopherectomy or treatment with androgens in NOD female mice (Fox, 1992; Hawkins et al., 1993; Fitzpatrick et al., 1991). Immune mechanisms affected by sex steroid hormones have not been determined. My data demonstrate a strong influence of estrogen and progesterone on MP PGS-2 expression and provide one potential mechanism for the impact of these hormones on IDD in NOD mice.

The enhanced expression of MP PGS-2 mRNA and protein during estrus phase suggests that female sex hormones regulate PGS-2 expression at both the transcriptional and translational level. These sex hormones could upregulate transcription or translation or enhance mRNA or protein stability. However, as PGS-2 mRNA is consititutively expressed during the non-estrus phase in female NOD mice, which suggests that other factor(s) may also play a role in the transcriptional induction of PGS-2 expression. These unknown PGS-2 regulatory factors in combination with a higher level of estrogen and progesterone during estrus phase could enhance PGS-2 mRNA and protein expression in NOD MPs.

It has been reported that the expression of PGS-2 in MPs is under strict control and regulation, and there are many complex autocrine regulatory interactions among different monokines and PGS-2 expression. Any defect in monokine expression could therefore affect PGS-2 expression, i.e., PGS-2 is induced by monokines such as TNF-α, IL-1 (Ristimaki et al., 1994), while it is suppressed by the monokines IL-10 and TGF-β (Mertz et al., 1994; Reddy et al., 1994). Defects in NOD monokines could therefore contribute to induction of PGS-2 expression through overproduction of stimulating

cytokines such as IL-1 and TNF-α from the NOD MP, or lack of function in factors that suppress PGS-2 expression such as IL-10 and/or TGF-β.

My data suggest that the level of TNF-α and IL-1 production by the NOD MP is similar to that of C57BL/6 and BALB/c MPs. These data suggest that aberrant PGS-2 expression in NOD MPs is not due to enhanced production of TNF-α and/or IL-1. To fully exclude the possible effect of TNF-α and IL-1 in the induction of PGS-2 expression, however, the sensitivity of PGS-2 expression to these cytokines needs to be tested by using TNF-α and IL-1 neutralizing antibodies in NOD MP cell culture.

The lack of IL-10 mediated suppression of NOD PGS-2 expression was unexpected result in these studies and provides important information as to a potential mechanism for PGS-2 dysregulation. Whereas 10 ng/ml recombinant murine IL-10 completely suppresses LPS induced PGS-2 protein expression in BALB/c and C57BL/6 MPs, concentrations of IL-10 as high as 500 ng/ml did not suppress spontaneous PGS-2 expression in NOD MPs. The mechanism responsible for the lack of IL-10 responsiveness in NOD PGS-2 expression could be mediated through a general desensitization of MPs to IL-10 in NOD mice mediated by a defect in the IL-10 receptor or post-IL-10 receptor signal transduction cascade. Alternatively, there may be differential regulation of IL-10 mediated suppression on PGS-2 expression. LPS induced PGS-2 expression is sensitive to IL-10 suppression; whereas, spontaneous PGS-2 expression in NOD mice whose protein expression is upregulated by sex steroid hormones may not be regulated by IL-10. Potential mechanisms are currently under investigation in our laboratory.

Inheritance of IDD and autoimmune susceptibility is under polygenic control in the NOD mouse. Approximately 14 NOD diabetes susceptibility loci have been identified and have been mapped to various chromosomes (Wicker et al., 1995). Chromosome 1 appears to contain at lease two insulitis susceptibility loci and one diabetes susceptibility locus (*Idd5*). PGS-2 located on chromosome 1 within *Idd5* interval. My data from two chromosome 1 congenic mouse strains supports a genetic basis for NOD PGS-2 expression. NOD.B10C1 is a NOD congenic mouse that contains a segment of chromosome 1 from C57BL/10snj mice including the C57BL/10snj PGS-2 gene and does not aberrantly express PGS-2 mRNA and protein. The replacement of an interval of NOD chromosome 1 with a C57BL/10snj segment delayed onset of diabetes to 14 weeks of age (onset on NOD is 10 weeks of age) and reduced the cumulative incidence of diabetes by the age of 30 weeks to around 40% (NOD is 80% at 30 weeks of age). Another congenic mouse strain we examined is the C57BL/6 congenic mice that contains a interval of NOD chromosome 1 (B6.NODC1) including the NOD PGS-2 gene on a C57BL/6 background. This congenic mouse develops peri-insulitis at 6 months of age. I demonstrated that MPs from B6.NODC1 mice constitutively express PGS-2. The differential expression of PGS-2 in NOD, congenic, and non-autoimmune control strains and its correlation with autoimmunity suggests it is a candidate susceptibility gene for *Idd5* that contributes to autoimmunity in NOD mice in concert with other susceptibility genes. However, because the existing congenic segments are large and contain many genetic loci, other genes within these intervals may affect the expression of PGS-2. To further define PGS-2 as a susceptibility gene for IDD, PGS-2 expression needs to be

examined in congenic mice that contain smaller intervals of chromosome 1 including or excluding PGS-2 gene and correlate the phenotype of PGS-2 expression with autoimmune disease in these animals.

To directly correlate the role of PGS-2 expression with diabetes, NOD female mice were treated with a combination of indomethacin (IN, 3ug/ml)/aminoguandine (AG, 0.1%) in their drinking water. This treatment significantly delayed the onset and reduced the incidence of diabetes by 40% compared to untreated mice. NOD mice treated with high doses (20ug/ml) of indomethacin alone have an identical effect on the incidence of diabetes as IN/AG treated NOD mice. These experiments suggest that enhanced PG production secondary to constitutive PGS-2 expression may impair immune tolerance in NOD mice and by blocking its enzymatic activity we can partly restore immune tolerance.

The mechanism regarding protection of NOD mice from diabetes by blocking PGS-2 expression is not yet known, but may function through effects on peripheral tolerance mechanisms. Multiple mechanisms are suggested to play a role in peripheral T cell tolerance. They include: 1) the generation of regulatory cells, 2) anergy, the antigen-specific loss of T cell proliferation and IL-2 production, and 3) activation induced cell death (AICD), the elimination of self-reactive T cells by activation with self antigen presented by APCs.

The important role of AICD in immune tolerance was suggested by *lpr/lpr* mice (Wu et al., 1994) that develop a lupus like autoimmune syndrome. Studies have

demonstrated that the defects in apoptosis of T cells in these mice directly contribute to the development of autoimmunity.

Some critical events for AICD include: TCR activation, lymphokine mediated cell cycle progression, IL-2 production, and TCR re-engagement (Guery et al., 1995; Stockinger et al., 1992). Studies have shown that PGE-2 can profoundly affect T cell functions that are very important for AICD. PGE-2 is a potent inhibitor of IL-2 and IL-2 receptor expression and can induce high levels of cAMP which blocks cell cycle in T cells (Goetzl et al., 1995; Lee et al., 1993; Ucker et al., 1994). It can also reduce the activation of PKC activity by interfering with the TCR signal transduction pathway proximal to PKC (Cook et al., 1994).

Since my studies have shown that there is enhanced PG production secondary to constitutive PGS-2 expression, I hypothesized that enhanced PGE-2 production impairs AICD in NOD mice and blocking PGS-2 enzyme activity may partly restore AICD in NOD mice.

By using SEB immunization as a model, AICD was found largely impaired in NOD mice. This experiment does not specifically address deletion of diabetogenic T cells, but reveals that NOD mice are characterized by a general AICD defect. After SEB immunization, I found Vβ8+/CD4+ T cells from C57BL/6 and BALB/c mice expanded at day 2 and deleted at day 10. In contrast, I found there was no Vβ8+/CD4+ T cells expansion at day 2 but a low level of deletion at day 10 in SEB immunized NOD mice.

These studies show that NOD H-2^{g7} plays a major role in the impairment of AICD, as NOD.H-2^{b} congenic mice, in marked contrast to NOD, show a nearly identical

expansion and deletion pattern as C57BL/6 mice. These data suggest that the unique H-2^{g7} MHC molecule plays a predominant role in the impairment of AICD in the SEB immunization model. The effect of H-2^{g7} on the impairment of SEB induced AICD may be secondary to its instability and decreased efficiency in presenting antigens (Carrasco-Marin et al., 1996).

However, pre-treatment of NOD mice with indomethacin and aminoguanidine (IN/AG) to block PGs production, doubles the deletion of Vβ8+/CD4+ spleen T cells on day 10; whereas, the same treatment has no effect on control BALB/c mice. These data suggest that enhanced PG production also contributes, but to a lesser degree than H-2^{g7} MHC gene to the impairment of AICD in NOD mice. However, blocking PG production does not increase expansion at day 2. The lack of an effect of this treatment on expansion could be due to several factors which include: 1) Addition of drugs to the animal's drinking water may not be an efficient way for drug delivery. As a consequence, enhanced PG production was not completely blocked and may still play a role in the suppression of T cell activation. 2) Intrinsic defects in T cell activation and in IL-2 and IL-2 receptor expression in NOD T cells play a dominant role in the suppression of T cell activation and this defect can not be reversed by blocking PGE-2 production. This may result in an impairment of AICD even in the presence of PG inhibitors, whereas the MHC can overcome these T cell defects and reverse impaired AICD.

In MPs, the aberrant expression of PGS-2 may have profound effects on monokine expression and macrophage function. In normal MPs, PGS-2 is only expressed following activation (e.g., LPS stimulation). Furthermore, its expression follows a

defined order of MP gene activation. PGS-2 normally follows TNF-α and IL-1 expression and precedes IL-10 expression. The order of expression of these monokines is critical to their expression and maintaining their regulatory interactions. In contrast to control MPs, PGS-2 in NOD MPs is spontaneously expressed prior to the induction of TNF-α and IL-1. The early expression of PGS-2 would potentially suppress both TNF-α and IL-1 while promoting IL-10 production. Therefore, PGE-2 induced suppression may partly explain the defects in TNF-α and IL-1 secretion described in NOD MPs (Serreze et al., 1993a).

Furthermore, PGE-2 production enhances IL-10 production and IL-10 in turn suppresses PGS-2 expression. Due to the NOD defect in IL-10 suppression of PGS-2, PGE-2 may enhance IL-10 production which may provide a potent negative effect on the function of MPs and other subpopulations of APCs. IL-10 has been shown to have a variety of biological effects *in vitro*. It is a potent inhibitor of macrophage function including cytokine synthesis, Iα expression, respirating burst, and PGS-2 expression (Mertz et al., 1994). In addition, IL-10 has been shown to suppress antigen-stimulated proliferation and cytokine production (IL-2, IFN-γ, TNF) by murine Th1 (CD4) T cells. IL-10 can also effect many of the same APC molecules PGE-2 effects as they both down-regulate MHC class II and B7 expression (de Waal Makefyt et al., 1991; Creery et al., 1994).

I find the absence of IL-10 suppression on PGS-2 expression in NODD MPs to be greatly interesting. The cumulative interaction of PGS-2, PGE-2 and IL-10 in NOD MPs allows for enhanced production of PGE-2 and IL-10. These two factors may lead to

further decrement of APC function in NOD mice. These possibilities are currently being explored in our laboratory.

CHAPTER 5
SUMMARY AND CONCLUSION

In this study, it was found that:

1. MPs from 8 week old NOD estrus female mice unlike those of non-autoimmune mice spontaneously express abundant PGS-2 mRNA and protein. PGS-2 is not normally expressed in control mouse strains, however, can be induced with LPS. Exposure of NOD MPs to LPS has little effect on the expression PGS-2 mRNA and protein. The expression of PGS-2, a MP early response gene, suggests that NOD MPs are activated and that the regulation of this gene in the NOD mouse differs substantially from that of control mice (BALB/c, C57BL/6, C57BL/10snj).

2. NOD scid/scid mice, like NOD mice, spontaneously express PGS-2 mRNA and protein. The expression of PGS-2 in the NODscid/scid suggests that functional lymphocytes and active autoimmunity are not required for MP expression of PGS-2 mRNA and protein. These data support the notion that PGS-2 is spontaneously expressed in NOD MPs and further suggests that NOD PGS-2 gene regulation differs substantially from control strains.

3. NOD estrus female mice express PGS-2 mRNA and protein whereas NOD males do not express this enzyme. Physiological concentrations of progesterone and estrogen induce PGS-2 expression in NOD male MPs *in vitro*. In contrast, neither *in vitro* androgens treatment nor castration of male mice *in vivo* alters the PGS-2 expression in

MPs of NOD male mice. These data demonstrate a sexual dimorphism in the expression of PGS-2 mRNA and protein in the NOD mouse. Because the expression of PGS-2 in male NOD MPs is strongly influenced by progesterone and estrogen, it suggests that the hormonal milieu explains the sexual dimorphism for PGS-2 expression.

4. PGS-2 expression in NOD MPs is insensitive to IL-10 as mrIL-10 (10ng/ml), which can completely suppress LPS induced PGS-2 expression in MPs of control mice, does not affect constitutively expressed PGS-2 in NOD MPs even at a concentration of 500 ng/ml. This study suggests a potential defect in the IL-10 receptor or post-IL-10 receptor signal transduction cascade. This potential defect may contribute to the dysfunction in suppression of PGS-2 expression in NOD MPs.

5. B6.NODC1 congenic mice contain an interval of NOD chromosome 1 which includes the NOD PGS-2 gene on a C57BL/6 background and spontaneously express PGS-2. In contrast, NOD.B10C1 congenic mice contain an interval of C57BL/10snj chromosome 1 that includes the C57BL/10snj PGS-2 gene and do not spontaneously express PGS-2. Data from these two congenic strains suggests a genetic basis for the NOD PGS-2 phenotype and makes PGS-2 a candidate gene for *Idd5*.

6. Treatment of NOD with a combination of indomethacin (3 ug/ml) /aminoguandine (0.1%) (I/A) or treatment of NOD mice with high does of indomethacin alone (20ug/ml) in their drinking water significantly delayed the onset and reduced the incidence of diabetes by 40% compared to untreated NOD ($p<0.03$). These data further support a critical role for PGS-2 in the pathogenesis of diabetes.

7. SEB immunization of NOD mice showed no Vβ8+/CD4+ T cells expansion at day 2 and only 10% deletion in those cells at day 10. These data suggest a general impaired activation and deletion of T cells in NOD mice compared with control strains (C57BL/6 and BALB/c). However, when NOD mice are pre-treated with I/A and immunized with SEB, there is a two fold enhancement of Vβ8+/CD4+ T cell deletion at day 10. The NOD MHC molecule H-2^{g7} plays a dominant role in the impairment of expression of NOD AICD as impaired AICD in NOD mice is almost fully restored in NOD.H-2^{b} congenic mice. This study suggests there is a general impairment of AICD in NOD mice which is predominantly affected by the MHC molecule and that PGS-2 contributes, but to a lesser degree than MHC, to the impairment of deletion.

Conclusion: Our data strongly suggest that spontaneous expression of PGS-2 in NOD MPs plays an important role in the immunopathogenesis of diabetes in NOD mice and is a candidate gene for *Idd5*.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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